SCREENING OF CASSAVA GENOTYPE FOR CASSAVA MOSAIC DISEASE RESISTANCE

By

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"SCREENING OF CASSAVA GENOTYPE FOR CASSAVA MOSAIC DISEASE RESISTANCE"** is a bonafide record of research 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, associateship, fellowship or other similar title, of any other University or Society.

Place: Sreekariyam

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LIST OF ABBREVIATIONS

%	Percentage
µl	Microliter
3’	Three prime
5’	Five prime
AAP	Acquisition access periods
ACMV	African cassava mosaic virus
ACMBFV	African cassava mosaic Burkina Faso virus
ALP	Alkaline phosphatase
bp	Basepair
CBSV	Cassava brown streak virus
CMD	Cassava mosaic disease
CMMGV	Cassava mosaic Madagascar virus
CMV	Cassava mosaic virus
CP	Coat protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTAB	Cetyl Trimethyl Ammonium Bromide
CTCRI	Central Tuber Crops Research Institute
cv	Cassava variety
DAPC	Discriminant Analysis of Principal Component
DAS ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
DIBA	Dot Immunobinding Assay
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dsDNA	Double stranded DNA
EtBr	Ethidium bromide
EACMCV	East African cassava mosaic Cameroon virus
EACMKV	East African cassava mosaic Kenya virus
EACMMV	East African cassava mosaic Malawi virus

EACMV	East African cassava mosaic virus
EACMZU	East African cassava mosaic Zanzibar Virus
ELISA	Enzyme Linked Immunosorbent Assay
F	Forward primer
FAO STAT	Food and Agriculture Organization Corporate Statistical Database
hr	Hour
ICAR	Indian Council of Agricultural Research
ICMV	Indian Cassava Mosaic Virus
IgG	Immunoglobulin G
IITA	International Institute of Tropical Agriculture
IR	Inverted Repeat
kb	Kilobase
M	Molarity
mAb	monoclonal antibody
MeSPY	Manihot esculenta Spindly
mg	milligram
min	Minutes
ml	millilitre
mM	Millimolar
MP	Movement Protein
ng	Nanogram
nm	Nanometer
NSP	Nuclear Shuttle Protein
OD 405	Optical Density at 405
ORF	Open Reading Frames
Ori	Origin of replication
PBST	Phosphate-buffered saline with Tween
PCR	Polymerase chain reaction
pH	Potential of hydrogen
pmol	Picomolar
PTGS	Post-transcriptional gene silencing

qPCR	quantitative PCR
R	Reverse primer
RCA	Rolling Circle Amplification
RCR	Rolling Circle Replication
REn	Replication enhancer protein
RNA	Ribonucleic acid
rpm	Revolutions Per Minute
RT PCR	Real time polymerase chain reaction
SACMV	South African cassava mosaic virus
sec	Second
siRNA	Small interfering RNA
SLCMV	Sri Lankan Cassava mosaic Virus
SNP	single nucleotide polymorphism
SPY	Spindly
ssDNA	single-stranded DNA
TAS ELISA	Triple Antibody Sandwich Enzyme Linked Immunosorbent Assay
TBIA	Tissue Blot immune Assay
TE buffer	Tris EDTA buffer
TGS	Transcriptional gene silencing
TME	Tropical *Manihot esculenta*
TMS	Tropical Manihot Selection
TrAP	Transcriptional Activator Protein
VIGS	Virus Induced Gene Silencing
ºC	Degree celsius
ϕ	Phi
β	Beta

1. INTRODUCTION

Starchy root and tuber crops play a major role in the human diet as well as in animal feed. They add variety to the diet by providing nutritional and health benefits. Tuber crops stand as a substantial part of the world's food supply. Nutritionally root and tubers have high potential to provide dietary energy in the form of carbohydrates.

Cassava (*Manihot esculenta* Crantz) is a staple tuber crop as well as subsidiary food which can grow in a wide range of climatic conditions, serving as the most important food security crop. Cassava realizes a better growth and a better development in deferent agro- ecological systems, even in the conditions presenting the poor fertility of soils with irregular rainfall (Thresh and Cooter 2005; Thresh, 2006). In India, cassava is grown in an area of 2.28 lakh ha with an annual production of 46.51 lakh tones (FAO STAT, 2018). Cassava is the fourth most important food source of carbohydrates in tropics. Due to the high starch content, it is the most predominant food for 500 million people (Blagbrough *et al.*, 2010).

Cassava mosaic virus is the major constraint to cassava production in the world. So far 11 species of cassava mosaic geminiviruses were identified from the African continent and Indian subcontinent. The infection of cassava mosaic virus leading 20-95% of yield loss has been reported among the countries across the world (Fauquet and Fargette, 1990). Cassava mosaic disease is transmitted mainly by infected stem cuttings and by whitefly (*Bemisia tabaci*) (Fauquet *et al*., 2005). For experimental purposes, mechanical transmission of the virus by grafting also showed a significant transmission of the virus to the virus-free plant.

The presence of cassava mosaic disease in India was first documented by Abraham (1956). The major cassava mosaic virus species that flourished in the Indian subcontinent include *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV). The diagnosis of the virus can be done using either PCR or by TAS ELISA (Makeshkumar *et al.,* 2005; Monde *et al.,* 2012). Cassava mosaic disease-

resistant plant can suppress the activity of the virus and thereby increase the yield. CMD causes severe mosaic, leaf distortion and stunted growth of plants, resulting in a considerable reduction in yields. The primary spread of the begomoviruses causing CMD is through the use of infected planting material and secondary spread in the field through whitefly transmission. But CMD has appeared in new areas essentially due to the inadvertent use of virus-infected planting material as even symptomless cassava plants, which are the usual source of planting material, may be infected (Malathi *et al.*, 1985).

The diagnosis of CMV was initially carried out with TAS-ELISA or by DAS-ELISA. The frequent occurrence of recombination in geminivirus led to more reliable PCR based detection. Coat protein gene (CP gene) specific primer can be used for the detection of viruses in India (Makeshkumar *et al.*, 2005).

Several control measures are available for the prevention of virus transmission including controlling vector by using chemical pesticides and eliminating virus-infected planting material. The use of virus-resistant plants showed the most significant control over the CMV spread. The cassava mosaic virus-resistant plant can suppress the activity of the virus and thereby increase the yield. A resistant plant can suppress the multiplication of the virus and consequently suppress the development of disease symptoms. Screening of cassava plants against cassava mosaic virus could provide resistant plants with higher productivity in on farm practices and could effectively used in breeding process. Whitefly inoculation, chip bud grafting and gene transfer methods are certain techniques used in resistance analysis. In this work chip bud grafting is preferred for resistance screening because of its inexpensiveness and effectiveness.

The objective of the present study is

- Screening of cassava plants for resistance to cassava mosaic virus using virus inoculation through grafting.

2. REVIEW OF LITERATURE

2.1 CASSAVA (*Manihot esculenta* Crantz)

Cassava (*Manihot esculenta* Crantz) is the most important vegetatively propagated food crop belongs to the family Euphorbiaceae. It is adapted to drought tolerance, low pH soils, varying climatic conditions with low management cost. It is originated in South America but now it becomes the major food source for more than 80 countries throughout the tropics.

Vegetative propagation by means of stem cutting is the widely used method for the propagation of cassava plants rather fertile seeds. The completely grown plant reaches 4 meters in its physiological maturity. Tuberous roots are reared 4 months to 6 years after planting. The tuberous root of cassava is the fourth most important reservoir of carbohydrate in the tropics with 32% to 35%.

Cassava roots are the abundant source of dietary material; additionally, they are prominent in industrial world with high economic value including bioethanol production and the paper industry. Except carbohydrate Calcium and vitamin C are rich in cassava tuber. Relatively protein content is very low in tubers but leaves are the rich source of methionine, cysteine, and cystine. Besides all cassava produce the largest carbohydrate content higher than rice and maize.

Asia is the second largest contributor to cassava production in the world after Africa. Asia shares 29% of the production share. In India, cassava is grown in an area of 2.28 lakh ha with an annual production of 46.51 lakh tones (FAO STAT, 2018).

The considerable production of cassava is used as food for humans, with lesser amounts being used for animal feed (Nwokoro *et al.,* 2002) and industrial purposes. Compared to other crops, cassava predominates under optimal conditions, offering the achievability of using marginal land to increase total agricultural production (Cock, 1982).

2.1.1 Threats in cassava production

Cassava is vulnerable to a diverse set of pests and diseases. Cassava is unprotected against at least 20 different viruses. Within that cassava mosaic virus and cassava brown streak virus are the economically important viruses which cause a serious reduction in yield (Legg *et al.,* 2006). Cassava mealybug (*Phenacoccus manihoti* Mat.-Ferr) and cassava green mite (*Mononychellus tanajoa* (Bondar) are the two other serious pest related to yield loss in cassava. Bacterial blight, viral diseases, root rots, whiteflies, mealy bug and cassava mites are other serious threats related to cassava production.

2.2 GEMINIVIRUSES

Gemniviruses are the group of plant infecting viruses belonging to the family Geminiviridae. They are circular ssDNA viruses with 2.5-3.0 Kb in genome size. The viruses present in the genera *Mastrevirus*, *Curtovirus* and *Topocuvirus* have single genomic component (monopartite), while those belonging to the genus *Begomovirus* have either one or two components (bipartite).

The largest genus within geminiviridae is *Begomovirus* which causes crucial losses in economically important crops. *Begomovirus* infects dicots and are transmitted by whitefly *Bemisia tabaci*. *B. tabaci* is a phloem-feeding insect and some biotypes have a very broad plant host range, including ornamental, vegetable, grain, legume and cotton plants (De Barro *et al.,* 2011). Circular ssDNA with bipartite genome is encapsulated with many copies of single coat protein (CP) subunit of 30 kDa.

The bipartite genome comprises two DNA segments, DNA A and DNA B, are each about 2.7 kb. Both segments have a role in infection. DNA A encodes six gene products: the coat protein (CP) (AV1) which also is the determinant of whitefly transmission, the AV2 protein which has a role in the viral movement, the Replicase (AC1) protein important for replication, Transcriptional Activator Protein (TrAP) (AC2)

that functions in viral ssDNA encapsulation and the suppression of gene silencing, Replication Enhancer (REn) (AC3), and AC4 protein which serves as an important symptom determinant. DNA B genes encode the Nuclear Shuttle Protein (NSP) (BC1) and the Movement Protein (MP) (BC2); both have a crucial role in the cell-to-cell movement of virus particles. The Open Reading Frames (ORFs) are organized bi-directionally in both genome components, separated by the IR which contains key elements for replication and transcription of the viral genome (Tennat *et al*., 2018).

CP is the only structural protein of geminivirus and it codes to form the viral capsid, other than that it has a role in insect transmission and shuttling of viral DNA into and out of the nucleus. Multi-functional protein Rep help in DNA replication by initiating (endonuclease activity) and terminating (ligase activity) (Rolling Circle Amplification). For that, Rep binds to dsDNA during origin recognition and introduces a nick in a highly conserved nonanucleotide (TAATATT↓AC) contained within a stem-loop structure that is part of the origin of replication. ORF AC4 is contained entirely within ORF AC1, but in a different frame. AC4 proteins are the least conserved of all geminivirus proteins, having diverse functions including virus movement and are involved in symptom development. In addition to their specific functions, AC4, TrAP and AV2 proteins have been shown to suppress transcriptional (TGS) and post-transcriptional gene silencing (PTGS), whereas Rep protein suppresses TGS. In the DNA-B, BV1 encodes the NSP required for trafficking viral ssDNA between the nucleus and the cytoplasm in the form of a viral DNA–NSP complex. For Cell-to-cell and long-distance movement, the NSP–viral DNA complex is trapped by the MP (encoded by BC1) in the cytoplasm and redirected to adjacent cells, where NSP directs the viral genome to the nucleus to initiate replication again (Zhao *et al*., 2019).

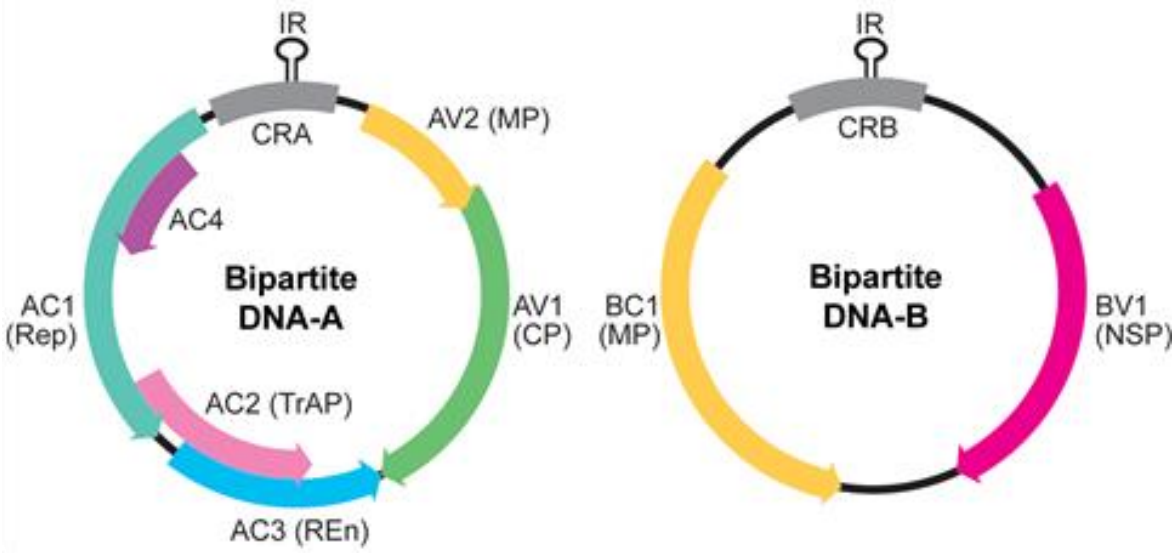

Fig. 2.1: Genomic organization of begomovirus [Brown, J.K. (2015)].

2.2.1 Replication

The replication of geminiviruses occurs in nuclei by rolling circle replication (Saunders *et al.,* 1991). The replication initiates after a cut was generated in the region named Ori (Origin of replication). Nick leads to the open up of 3'OH which serves as the primer during DNA synthesis. Various DNA forms detected upon *African cassava mosaic virus* help to identify the rolling circle mechanism as the mode of geminivirus replication (Saunders *et al*., 1991). ssDNA is introduced into the plant cell by the feeding of the insect. Host factors (DNA polymerase) help to convert ssDNA (+ strand) to dsDNA intermediate replicative form. This replicative form serves as a template for viral replication as well as its bidirectional transcription.

During the second stage of replication Rep (AC1) produces a nick on the (+) strand of the replicative form. The nick is created at the highly conserved specific nonamer sequence (TAATATT↓AC) present in the loop region of the stem-loop structure of the circular DNA molecule (Laufs *et al.*, 1995). Pasumarthy *et al.* (2010) identified that, in order to enhance replication; Rep interacts with another viral protein REn (replication enhancer protein encoded by AC3). Later another nick is produced by Rep

and it transferred to another 5’ terminus. Rep acts as ligase finally to produce a circular ssDNA molecule.

During the final stage of RCR, new viral progenies are produced by encapsidation of newly synthesized ssDNA molecules. Later ssDNA molecules are converted to dsDNA molecule. Sunter *et al.* (1993) identified AC2 (TrAP) and AV2 mutants were found during the dsDNA synthesis. The spread of virus was done by host’s transport machinery. The nuclear shuttle protein (NSP encoded by BV1) and the movement protein (MP encoded by BC1) of the virus helps in establishing viral infection. Cell to cell movement of the virus is established through plasmodesmata and the vascular system provides long- distance movement.

2.3 CASSAVA MOSAIC DISEASE

Cassava mosaic disease is the major hindrance in the cassava production, caused by begomovirus. Cassava mosaic disease in Africa and the Indian subcontinent is caused by one, or a mixture (Legg and Fauquet, 2004; Legg *et al*., 2006) of the eleven species of distinct cassava mosaic geminiviruses (CMGs). Of the begomoviruses associated with CMD, *Indian Cassava Mosaic Virus* and *Sri Lankan Cassava mosaic Virus* are the major two which flourished in Indian subcontinent. All these viruses are transmitted by *B. tabaci* (Maruthi *et al.*, 2014), and the disease is spread through the use of infected planting materials (Malathi *et al*., 1989). In India, CMD was recognized as a major threat to the cultivation of cassava in the early 1940s (Abraham, 1956). CMD causes severe mosaic, leaf distortion and stunted growth of plants, resulting in a considerable reduction in yields.

Interspecific recombination between monopartite and bipartite begomoviruses leads to the arise of ACMV- like recombinant begomovirus, sharing 93% nucleotide identity with DNA B of ACMV. The detection of ACMV like recombinant begomovirus confirmed recombination plays a major role in infection of cassava (Tiendrebeogo *et al*., 2012).

2.3.1 Types of Cassava mosaic virus

Cassava mosaic disease is one of the major obstacles in cassava production. It severely affects the cassava production in African and Asian continents.

Eleven species of cassava mosaic geminivirus species (genus: Begomovirus, family: Geminiviridae) include;

1. *African cassava mosaic virus* (ACMV)
2. *African cassava mosaic Burkina Faso virus* (ACMBFV)
3. *Cassava mosaic Madagascar virus* (CMMGV)
4. *East African cassava mosaic Cameroon virus* (EACMCV)
5. *East African cassava mosaic Kenya virus* (EACMKV)
6. *East African cassava mosaic Malawi virus* (EACMMV)
7. *East African cassava mosaic virus* (EACMV)
8. *East African cassava mosaic Zanzibar Virus* (EACMZV)
9. *South African cassava mosaic virus* (SACMV)
10. *Indian cassava mosaic virus* (ICMV)
11. *Sri Lankan cassava mosaic virus* (SLCMV)

(Patil and Fauquet, 2009, Legg *et al.*, 2015 and Fondong, 2017)

2.3.2 Global prevalence of cassava mosaic disease

African cassava mosaic virus is the most widely distributed pathogen that causes extreme yield loss in cassava in Africa. The first report of ACMV from Tanzania was reported by Warburg (1894). Further analysis of ACMV plants which were affected in Kenya revealed the presence of another virus that is similar to ACMV in the genomic organization with distinct serological properties. The presence of EACMV was confirmed by Hong *et al.*, 1993. Of all characterized viruses, 7 of them are isolated from sub-Saharan Africa.

The cassava mosaic disease has been reported from Africa and the Indian sub-continent. Now it is being flourished to many south East Asia as it was reported in China, Vietnam, Cambodia, and Laos (Marquie and Reynaud, 2019). The presence of *Sri Lankan Cassava Mosaic virus* was confirmed by Wang *et al.* (2019) by PCR analysis was performed using SLCMV specific primers AF/R. The spread of cassava mosaic disease in Uganda was reported by Legg and Thresh (2000) during the survey conducted 1990's. The spread of disease leads to the massive destruction of the crop and thereby it challenged the food security. A novel recombinant virus which is similar to ACMV and EACMV with interspecific hybridization was reported in Uganda with probable role of recombination (Zhou *et al*., 1997).

The presence of EACMKV was reported in Kenya by Bull *et al.,* (2006) as novel begomovirus with full length sequence of 109 components (68 DNA-A and 41 DNA-B) consisting similarity with *East African cassava mosaic virus* and *East African cassava mosaic Zanzibar virus*. There exists large variation in phenotypic symptom for each of these virus isolates, irrespective of their location (Bull *et al.,* 2007).

2.3.3 Cassava mosaic disease in India

Cassava is majorly grown in southern states of India including Kerala, Tamil Nadu, Andhra Pradesh and also some eastern states. In India cassava is grown in an area of 2.28 lakh ha with an annual production of 46.51 lakh tones (FAO STAT, 2018).

Abraham (1956) was the one first documented the presence of cassava mosaic disease in India, later it was elaborated by Alagianagalingam and Ramakrishnan (1966). Austin (1986) identified similar disease caused by different begomovirus in Sri Lanka, which was letter identified as *Sri Lankan cassava mosaic virus* (Saunders *et al*., 2002).

The first clone of *Indian cassava mosaic virus* (ICMV-Ker) was obtained by Hong *et al.* (1993) and subsequently, Saunders *et al.* (2002) cloned SLCMV. The DNA-A and DNA-B components of ICMV share 65% and 30% nucleotide sequence similarity with ACMV DNA-A and DNA-B, respectively. Although SLCMV has similar iteron

sequences to those of ACMV, it shares a greater identity with ICMV. The differentiation of viruses based on symptom is not possible.

Survey conducted by Anitha *et al.* in 2011 concluded that the presence of *Sri Lankan cassava mosaic virus* is widespread in Kerala.

2.3.4 Plant host range

Cassava mosaic virus is the most important single factor that could result in the major yield loss in the cassava. *Manihot esculenta* is the most common host for cassava mosaic virus. ACMV is widely restricted to the Solanaceae family but within it showed chlorotic local lesion on certain species of Nicotiana like *N. benthamiana, N. clevelandii* and *Datura stramonium* (Jose *et al.,* 2008). Solanaceous hosts including *Datura stramonium* and different species of *Nicotiana* showed SLCMV positive (Anitha *et al.,* 2011) with chlorotic spots, curling of leaves, leaf distortion, reduction in leaf size, mosaic, stunting and vein clearing. Experimental host range of SLCMV and ICMV is seems to be higher. Host range of ICMV was reported to be transmitted to the 43 species of *Nicotiana* (Mathew and Muniyappa, 1993).

Jose *et al.* (2008) performed sap inoculation of SLCMV. Among the plant inoculated 39 species in Solanaceae family were positive for SLCMV infection. The transmission of ICMV from cassava to cassava through whitefly was reported by Antony *et al.,* 2006 besides it has role in transmission of cassava to cucumber by whitefly (Menon and Raychaudhuri, 1970; Mathew and Muniyappa, 1993). ACMV was reported to be transmitted to 13 species by sap inoculation (Bock and Guthrie, 1978; Walter, 1980).

The host range of EACMV as well as SACMV has not been studied extensively, but it is known to infect *Nicotiana* species, especially for the commonly used experimental host *N. benthamiana* (Berrie *et al.,* 2001). Major hosts of ICMV include *Nicotiana* spp, *Petunia hybrid* and *Nicandra physoides*. The presence of virus was confirmed by the symptoms including leaf curling, crinkling and chlorotic lesion, leaf

deformations which were produced 6–10 days after inoculation (Mathew and Muniyappa 1993).

2.4 SYMPTOMATOLOGY

Occurrence of characteristic mosaic patterns over the infected leaves was first observed by Storey and Nichols (1938). Mosaic patterns in the plants can be determined in the very early stage. Symptoms vary from leaf to leaf or plant to plant according to the different virus strain, plant adaptation, temperature difference or even plant age. Chlorotic mosaic of the leaves, leaf distortion, and stunted growth are some of the infected leaf characters (Legg and Thresh, 2000).

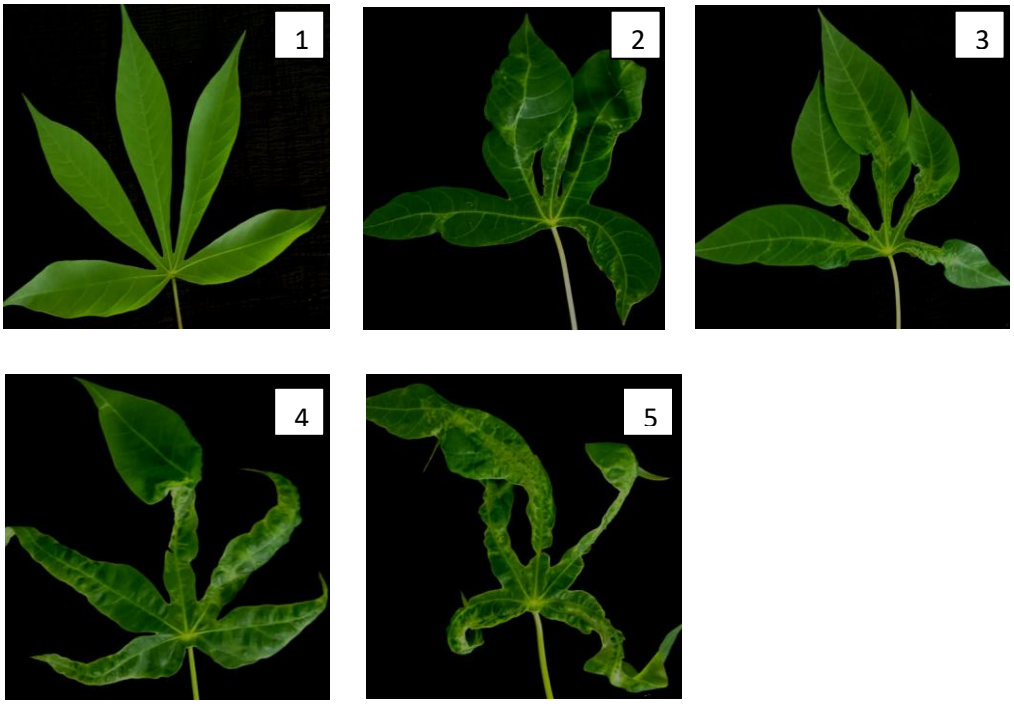

Fig. 2.2: The cassava mosaic symptom scale (1-5). 1) Unaffected shoots with absence symptoms 2) Mild chlorosis, mild distortions at bases of most leaves 3) Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets 4) Severe mosaic distortion of two thirds 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 (Hahn *et al.*, 1980).

The chlorotic areas appeared on the leaves are usually clearly defined and it vary the size from a whole leaflet to small blotch. Localized mosaic spots as well as distortion, reducing the leaflet size along with stunted growth are associated with symptom severity. Some plants situated in the affected one shows recovery nature. It seems to the influence of ambient temperature and the host resistance. But the symptom seems to be reappearing in the recovered plants as the environmental condition change (Gibson and Otim-Nape, 1997). Sometimes the first few leaves produced by the infectious cuttings are symptomless, but are subsequently followed by the severely affected leaves. Still there is a chance for the fall off of symptoms as plants age especially for the resistant varieties.

2.5 TRANSMISSION

Cassava mosaic virus transmitted mainly by infected stem cutting and whitefly *Bemisia tabaci* (Chant, 1958, Dubern, 1994). Experimentally they can also transmit mechanically to some Solanaceae family. Dissemination through the infected stem cutting is the inevitable consequence of the vegetative propagation of cassava leads to overall distribution of virus. Non validated stem cuttings due to the high demand of the planting material as well as the lack of functional seed systems increased the spread of viral disease (Mukiibi *et al*., 2019). Another mean of transmission is through whitefly vector, *B. tabaci.* Begomoviruses are known to be vectored by the whitefly *B. tabaci*, a species consisting of more than 36 genetically distinct but morphologically 81 indistinguishable cryptic species (De Barro *et al.,* 2011; Liu *et al.,* 2012). By mechanical inoculation and by means of Agro inoculation (*Agrobacterium* mediated transfer) from partially or tandemly repeated cloned genomic DNA or biolistic delivery of cloned genomic DNA for experimental purpose also shows transmission of viral particles (Rojas *et al.,* 2005). However, CMD is not transmitted through seeds (Mathew, 1989; Makeshkumar *et al.,* 2005)

2.5.1Whitefly

Whiteflies serve as the most important vector for the economically destructive plant viruses. Among this, whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) affect more than 600 crops and weed hosts. It is a vector of 70 plant-infecting viruses in tropical and sub-tropical countries (Brown *et al.* 1995; Perring, 2001). Among all these viruses more than 80% of the known geminiviruses are transmitted by whiteflies and belong to the genus Begomovirus (Varma and Malathi, 2003). The whiteflies mainly feed the plant tissues by sucking the cell sap. They are major vectors for disease transmission rather being a pest. Antony *et al.,* (2006) detailed that *Bemisia tabaci* has major role in transmission of ICMV from cassava to cassava. *B. tabaci* adults from colonies reared on cassava or sweet potato plants were studied to determine their ability to transmit ICMV from cassava to cassava. Virus acquisition access (feeding) periods (AAP) of 48 h was given for whitefly reared on ICMV infected cassava leaves and ICMV was successfully transmitted from cassava to cassava by whiteflies.

2.5.2 Grafting

Graft inoculation is the one of the most widely used experimental means of transmitting viral infection. The time needed for the successful grafting may varies by several days to months. Scion is the separated shoot portion of the plant and root bearing portion is known as the stock. Subsequent infection of scion or stock leads to the infection of whole plant (Zaltlin, M., 1991 and Nayudu, 2008). Formation of callus produced by cambium layer on both scion and stock indicate success of grafting. Compatibility of scion and stock will lead to the success of graft. There are many types of grafting is available. Environmental conditions have great influence on grafting; usually they are kept in humid conditions to prevent the water loss. Some commonly used methods of grafting are explained below.

2.5.2.1 ***Approach grafting*** (Li *et al.,* 1996)

In this method two entire plants are brought together and grafted. Diseased and healthy plants are brought together in two different pots. Make a long cut on the cambium

portion of both plants at same length. Tie them together until the establishment of graft. Graft failure due to the dehydration can be minimized by this method.

2.5.2.2 *Wedge or Top Cleft Grafting* (Harris, 1932)

For this method of grafting a slit is completely made through the stem of stock plant and a scion with “V” shaped with similar size of the stock plant is inserted into the cleft. After cutting the stock plant a longitudinal incision is created at the top of the plant. The edges of the scion are cut since to be getting attached with the cambium. After fixing them perfectly place a wet cotton swab on the cut region in order to avoid dehydration.

2.5.2.3 *Tongue Grafting* (Nam and Kim. 2002)

The tongue grafting is preferred when dealing with soft stem. Tongue shaped incision is created in stock and scion. A downward slit is created in the stock plant and an upward slit is on the scion with sharp scalpel blade. Remove epidermis of each graft. Grafted tissues are bound tightly with wet tissue. Try to keep the moisture control of the plant to get successful grafting.

2.5.2.4 *Bud (Shield) Grafting* (Shalla *et al.,* 1964)

In this method scion having single bud is used for lateral grafting. The bud (scion) is removed with a few surrounding tissues and it is inserted into the stock plant by removing bark of the plant in “T” shape. In the “T” shaped insertion, two bark flaps are lifted and the bud is inserted into the flap.

2.5.2.5 Side Grafting (SIG) (Mohamed *et al.,* 2014)

In this method young stock and scion is used for grafting. The stock and scion were selected with matching length. The growing part of stock was removed carefully. Then 35º to 45º angle cut was carefully done on selected scion plant. The scion was then inserted into the slit in the rootstock. The two cut surfaces were matched together and held with a grafting clip. Grafted plants were then transferred to a humidity chamber.

Plants were maintained in the greenhouse until the scion was connected well to the rootstock.

2.5.2.6 *Chip Bud Grafting* (Wagaba *et al.,* 2013)

In this method axillary bud from the virus infected plants are used as the scion. In this method stem portion is slightly removed from the stock plant since to open up the cambium tissue. The axillary bud excised from the virus infected plant with petiole is inserts into the healthy plant. Bud graft is secured using a parafilm. Callus formation seen within 1 week and virus transmission takes place within 2-6 weeks.

2.6 RESISTANCE SCREENING IN CASSAVA

Evaluation of resistance cassava mosaic diseases in selected cultivars of cassava in Africa was studied by Houngue *et al.,* (2018) showed effective result by grafting method. Combined molecular as well as grafting tools are used for the resistance study. The result can be reliably used for the selective production and conservation of germplasm of cassava. Resistant lines without *African cassava mosaic virus* can be effectively raised by grafting method. The technique is very effective for rapid selection of cassava varieties against cassava mosaic disease with an exceeding success rate of 65%.

Screening of cassava mosaic begomoviruses resistance using grafting and by whitefly inoculations were carried out by Monde *et al.,* (2012). In this study, *East African cassava mosaic virus* transmission is analyzed by grafting and whitefly inoculation. The cultivar which does not show any CMD symptoms were selected for the resistance screening programme. The severely infected cuttings (scion) were grafted on the resistance lines and maintained well to know the resistance. The approach used for the resistance screening is by whitefly inoculation. The CMBs free whiteflies are reared and inoculated to the infected plant to get the virus load in whiteflies. The virus load was evaluated by PCR and TAS ELISA. The confirmed vectors are used for resistance

studies. For resistance cultivars the grafting and whitefly inoculation has no effect in the manifestation of CMD. So, it can be usefully recommended for farmers.

Top- cleft grafting is another approach for grafting which was done by Anjanappa *et al.,* (2016) for *Cassava brown streak virus* resistant cassava production. Natural CBSV is the key to control CBSV in Africa, because it has become the major constraint to the cassava production. The plants are assessed by their viral resistance by top- cleft grafting. After the 14 weeks of successful grafting side grafting was carried out as double- grafting procedure to know the resistivity of the lines. Successful lines are detected by these methods with symptomless cultivars which do not support virus replication and accumulation.

Wagaba *et al.,* (2013) performed efficient transmission of cassava brown streak virus by chip bud grafting method. In this study axillary buds are collected from the cassava brown streak diseased plants with virulent isolates of *cassava brown streak virus* and *Ugandan cassava brown streak virus*. The isolated buds are inoculated into healthy disease-free plants by chip bud grafting. The symptom development is assessed visually and then confirmed using RT-PCR. Bud grafting is an improved tool for the transmission of viruses with a high success rate. In this type of grafting test plants can be inoculated in young stage and that can be handled well in greenhouse. The chip bud grafting allows rapid symptom development and there by better studies.

Evaluation of cassava varieties for cassava mosaic disease resistance by agro inoculation and by molecular markers were carried out by Bi *et al.,* (2010). The selected varieties collected from the fields of China, Thailand and other Asian countries were analyzed for the resistance study. Clones of *A. tumefaciens* strain LBA4404 containing the infectious clones of ACMV-NOg DNA-A and DNA-B (Vanderschuren *et al.,* 2009) are used for the infection study to know the resistance. From the selected cassava lines all tested lines were shown positive for CMD and none of them were shown resistance to ACMV.

Evolution of virus resistance screening takes 12-18 months under field condition. While in greenhouse practices resistance evaluation is done by transmission of virus from infected source to healthy plants by grafting or *Agrobacterium* mediated or by using biolistic delivery of infectious clones. Still it seems to be requiring 12-22 weeks. VIGS (Virus Induced Gene Silencing) is another rapid screening system to check resistance and susceptibility to CMD. The VIGS vector was developed based on virulent strain *East African cassava mosaic virus.* Arabidopsis *SPINDLY* (*SPY*), sequence from the cassava (*Manihot esculenta*) ortholog was cloned into the CP position of the DNA-A genomic component. Silencing of *M. esculenta* SPY (*MeSPY*) using MeSPY1-VIGS resulted completed death of Susceptible plants by severe shoot tip Necrosis within 2-4 weeks (Beyene *et al.,* 2017*).*

Carmo *et al.,* (2015) used Molecular-assisted selection for resistance to cassava mosaic disease in *M. esculenta Crantz*. Five molecular markers (NS169, NS158, SSRY028, SSRY040 and RME1) that were associated with resistance to CMD, along with 402 SNPs (single-nucleotide polymorphism) were used for the genotyping. Potential sources of resistance to CMD were identified by the *CMD 2* associated accessions with flaking markers (NS169+RME1, NS158+RME1 and SSRY28+RME1).

2.7 ECONOMIC IMPACT

Cassava mosaic disease is the most important disease for cassava which causes high yield loss. Yield loss of the disease depends upon the varieties being affected. In the case of ICMV and SLCMV yield loss is upto 45 % reduction for susceptible varieties (Malathi *et al.*, 1985). 20%- 95% yield loss have been reported from individual countries across the world (Thresh and Otim Nape,1994). CMD cause chlorosis in the leaf which eventually leads to the reduced photosynthetic rate and hence it will lead to large variation in the yield (Chikoti *et al.,* 2019).

Mixed infection of ACMV and EACMV- UG caused higher yield loss in Uganda up to a percentage of 12-82%. The plant only affected with mild strain EACMV- UG

showed yield loss for only 12% when comparing with ACMV infected plants; it was reduced by 42%. Severe strain of EACMV- UG showed yield loss of 68%. The mixed infection of both strains increased the yield loss to 82% (Owor, 2002).

The CMV resistance breeding lines could facilitate the enhanced production of tubers and thereby can control the yield loss.

2.8 DETECTION AND DIAGNOSTICS

Cassava mosaic diseases are affected by different types of begomoviruses under the family Geminiviridae. In Indian subcontinent *Indian cassava mosaic virus* and *Sri Lankan cassava mosaic virus* are the two major viruses which cause CMD. PCR based detection, ELISA based detection are some methods related with detection of viruses. Other than these methods, sequence based analysis provides most accurate detection of CMD.

2.8.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is a simple method for the detection of specific viruses with amplification of target sequence. Melting of the target, annealing the denatured strand, primer extension using Taq DNA polymerase are the three essential steps involved in PCR. Effective use of PCR helped in the detection of viruses and there by diagnosis (Henson and French, 1993). The efficiency as well its productivity hang on with concentration of dNTP, type of polymerase, purity of template and cycling parameters (Lopez *et al.,* 2006). Along with other techniques it is considered as the standard tool in diagnosis.

2.8.1.1 *Multiplex PCR*

Concurrent detection of two or more target pathogenic DNA or RNA molecule by using several specific primers in single PCR reaction is possible with Multiplex PCR. Different formats of PCR and multiplex PCR have been applied for the detection of ICMV and SLCMV (Anitha *et al.,* 2011; Dutt, *et al.,* 2005; Hegde *et al.,* 2010;

Makeshkumar *et al.*, 2005). EACMV and ACMV in Africa (Alabi *et al.*, 2008) as well as SLCMV and ICMV in India can be detected by using multi PCR (Patil *et al.*, 2005).

2.8.1.2 *Real- time PCR*

Accuracy of the PCR products can be confirmed using RT- PCR (Heid *et al.*, 1996). The running process of the real-time PCR can be monitored directly by the exponential curve analysis on a computer screen (Gibson *et al.*, 1996). Cassava mosaic viral infection with SLCMV can also be detected as well as quantified using real time PCR (Deepthi *et al.*, 2012).

2.8.2 ELISA

ELISA- Enzyme Linked Immuno sorbent Assay is a diagnostic method used for the detection of antigen by using a specific antibody. Use of polyclonal and monoclonal antibodies make ELISA technique is more sensitive (Pankova *et al.*, 2002). Cassava mosaic geminiviruses in Africa is mainly determined by using DAS- ELISA and TAS-ELISA where TAS- ELISA is more effective than DAS-ELISA. It can be used for viral detection as well as quantification of viral load in the sample. It is one of the most sensitive methods for detection of plant viruses in large scale.

A single-tube duplex and multiplex PCR for simultaneous detection of four cassava mosaic begomovirus species in cassava plants was developed by Aloyce *et al.*, (2013). It helped for the simultaneous detection of *African cassava mosaic virus* (ACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV) and *East African cassava mosaic Zanzibar virus* (EACMZV). Duplex and multiplex enabled the simultaneous detection of four CMBs, namely ACMV (940 bp), EACMCV (435 bp), EACMMV (504 bp) and EACMZV (260 bp) in single and mixed infections.

Rolling circle amplification is an innovative approach utilizes the ϕ29 DNA polymerase. The divergent genetic variability of cassava mosaic diseases can be confirmed by using rolling circle amplification followed by sequencing. Help for the

clear detection of mixtures of isolates, strains, and multiple species within the sample. RCA increased the chances for detecting the viruses (Kathurima *et al.,* 2016).

Several serological approaches have been done for the detection of ICMV including ELISA, DIBA, TBIA (Makeshkumar and Nair, 2001; Malathi *et al.,* 1985; Malathi *et al.,* 1989). SLCMV and ICMV are detected by several PCR analyses as well as by multiPCR methods (Makeshkumar *et al.,* 2005). For large number of sample analysis Nucleic acid spot hybridization has major role and it has been done in India by Makeshkumar *et al.,* (2005) for the CMG detection.

TaqMan-based real-time PCR assay (qPCR) for the simultaneous detection and quantification of two RNA viruses; *Cassava brown streak virus* (CBSV) and *Uganda cassava brown streak virus* (UCBSV) and two predominant DNA viruses; *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV). Real Time analysis is sensitive than conventional PCR and thus a more precise tool for quarantine and resistance screening purposes (Otti *et al.,* 2016). Real time PCR method has been developed for the detection of very small amount of SLCMV by Deepthi *et al.,* (2012).

2.9 CONTROL MEASURES

Virus free plant from an infected plant can be obtained using the meristem tip culture technique, which showed 80% efficiency in obtaining virus free plant (Adejare and Couts, 1981). Virus can spread rapidly by virus infected stem cutting. Effective removal of diseased plants from the standing crop can reduce the infection spread (phytosanitation). Resistant variety has less potential to transmit infection; they are poor source of inoculum so the vectors can't transmit the disease (Thresh *et al.,* 1998).

Vector population like whitefly which transmits the virus can be preventing by use of pesticides or other chemicals by decreasing vector population or by interrupting its transmission. Transgenic approaches have gained new approaches for creating virus free plants (Vanderschuren *et al.,* 2012) to generate transgenic cassava lines a hairpin

construct was transferred to cassava lines by agro bacterium mediated transformation. Combining resistance for CMV and CBSV was created by agro bacterium mediated transformation. This innovative combination could able to effectively control the EACMV.

A hairpin-RNA construct targeting *South African cassava mosaic virus* AC1/AC4 overlapping genome region confers tolerance in cassava. Transgene derived RNA hairpin showed tolerance in the CMD-susceptible model cassava cultivar 60444. Expression of SACMV AC1/AC4 homologous siRNAs resulted that tolerance is most likely associated with post-transcriptional gene silencing of the virus (Walsh *et al.,* 2019).

Molecular-assisted selection for resistance to CMD is one of the successful strategies used for the identification of resistance varieties and for identifying favorable crosses in breeding (Carmo *et al.,* 2015). The accessions were analyzed by using molecular markers (NS169, NS158, SSRY028, SSRY040 and RME1) that were associated with resistance to CMD, along with SNPs (single-nucleotide polymorphism). The promising crosses were identified using discriminant analysis of principal component (DAPC), and the matrix of the genomic relationship was estimated with SNP markers.

Whiteflies are the key pest during the open field cultivation of cassava. Effective vector control by use of several insecticides to control the vector can effectively be implemented for the disease control.

Now new advancing techniques like CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) going on to create new opportunities to confer effective resistance to the virus.

2.9.1 Resistance varieties for controlling CMD

Cassava breeding is the most successful control for cassava mosaic disease resistance. A successful cross was obtained by crossing of wild cassava, *Manihot glaziovii* into cultivated cassava. Legg and Thresh (2000) confirmed the CMD resistance nature of *M. glaziovii* and it is designated as CMD1. The monogenic CMD resistance

locus, CMD 2 identified by Akano *et al*., (2002) in some West African cassava landraces, referred to as tropical *M. esculenta* (TME). International Institute of Tropical Agriculture (IITA) in collaboration with Cameroon introduced the five varieties foe CMD resistance analysis. They showed CMD incidence of less than 10% in resistance lines with CMD1 and CMD 2 resistance. Among that TMS 96/0023 displayed a strong resistance to CMD (Fondong, 1999). Zang *et al.* (2005) developed transgenic cassava which is resistance to ACMV using improved antisense RNA technology by targeting the viral mRNAs *Rep* (*AC1), TrAP* (*AC2)* and *REn* (*AC3).* Expressing antisense RNAs against viral mRNAs could effectively reduce the virus load. Walsh *et al.* (2019) developed transgene derived RNA hairpin, homologous to an overlapping region of the SACMV replication associated protein for resistance analysis. Expression of SACMV AC1/AC4 homologous siRNAs showed that this tolerance is most likely associated with post-transcriptional gene silencing of the virus for CMD-susceptible model cassava cultivar 60444.

2.9.2 CMD Resistance in India

Several analyses are going on for the development of SLCMV and ICMV resistance lines. Sree Padmanaba (TMS30001 line Mnga-1 line), 242 resistant lines from open pollinated seedlings of Mnga-1, two of three lines CMR-1 and CMR 129 have been successfully analyzed in field conditions and released for cultivation (Unnikrishnan *et al.*, 2011). *M. caerulescens* showed a higher level of resistance when several interspecific hybrids were generated, the hybrid donors used as introgress the genes into elite Indian cultivars (Sheela *et al.,* 2012). For resistant line selection crossing between 9s- 75 x CI-273 provides a maximum number of resistant lines. 16s- 203 produced higher tuber yield within the resistant lines. Another study with a reciprocal cross made with TMS-96/1089A x CR 43- 11 and TMS-30572x CR 43-11 were analyzed for the resistance study. From this 325 seedlings were analyzed for the presence of *cmd1* and *cmd 2* genes. 150 seedlings were shown resistance during the analysis of CMD associated SSR marker. On field trials they showed 100% field resistance to CMD (Annual report CTCRI, 2017).

3. MATERIALS AND METHODS

The study entitled “Screening of cassava genotype against cassava mosaic disease resistance” was carried out at the Division of crop protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2019-2020. Details regarding to the experimental material and procedures used in the study are elaborated in this chapter.

3.1 COLLECTION OF INFECTED SOURCE PLANT AND ESTABLISHMENT IN GLASS HOUSE AS VIRUS INOCULAM SOURCE

The cassava mosaic disease (CMD) samples were collected from the fields in ICAR- CTCRI, Thiruvananthapuram and from the fields near Vizhinjam. The leaf samples were collected and symptoms were recorded as described by Hahn *et al.* (1980). The infected leaf samples were collected, properly labeled, covered with polythene bag and stored at -80ºC for further analysis. The infected stem cuttings were planted in the glass house. The plants were maintained well for further analysis.

3.2 CONFIRMATION OF VIRUS INFECTION THROUGH PCR

The presence of infection in the source plant was confirmed using PCR method. Multiplex PCR is used for the detection of virus load in the samples.

3.2.1 DNA isolation

CTAB method described by Lodhi *et al. (*1994) was used to isolate the total DNA from infected plants. β-mercaptoethanol was added fresh to the CTAB extraction buffer (Appendix I) to give a final concentration of 2% (v/v). The buffer was pre-heated to 60ºC in water bath. 100 mg of plant samples were weighed and ground to a fine powder with liquid nitrogen using a sterile mortar and pestle. Then transferred the contents in to a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion and then incubated at 60°C in a water bath for 30 min with intermittent shaking. Later it was centrifuged at 10,000 rpm for 10

min at 4ºC. The supernatant was transferred to another sterile centrifuge tubes with a sterile pipette tip. To this 10 µl activated RNase (10 mg ml^{-1}) and 10 µl Proteinase K (20mgml^{-1}) was added and incubated at 37°C for 1 hr. The homogenate was then extracted with an equal volume of 25: 24: 1 (v/v) phenol:chloroform:isoamyl alcohol (Appendix I) and mixed well by inversion until it turns milky white. The contents (Hermle, Table- top refrigerated centrifuge) were centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was then extracted with an equal volume of 24: 1 (v/v) chloroform:isoamyl alcohol (Appendix I) and mixed well and centrifuged at 13000 rpm for 10 min at 4°C. To the aqueous phase, 0.8 volume of chilled isopropanol and 1/10 volume of 3 M sodium acetate with pH 5.2 was added and mixed by inversion. The mixture was then incubated at -20ºC for at least 1 hr or overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 13000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (70 %) and centrifuged at 13000 rpm for 10 min at room temperature. Again the supernatant was discarded and the pellet was air-dried for 30-40 min and dissolved in 50 µl of TE buffer (Appendix I) or deionised water. The extracted DNA samples were then stored at -20ºC.

3.2.2 Agarose gel electrophoresis

The quality of the DNA extracted by CTAB method was analyzed by using Agarose gel electrophoresis. An agarose gel of 0.8% was prepared using 1X TAE buffer (Appendix II). 0.5 µll^{-1}ethidium bromide (EtBr) was added to the solution. Approximately 2µl of isolated DNA along with gel loading dye was loaded into the wells. The gel was run at 70v for 30 minutes. Then the gel was visualized under UV light and the image was captured using Azure gel documentation system. The quantity of DNA was analyzed using a spectrophotometer (Denovix DS 11+ spectrophotometer).

3.2.3 Polymerase chain reaction

In order to identify virus present in the plant for grafting purpose was carried out by using the polymerase chain reaction. The synthesized primers of 100pM concentration were diluted to 10pM with sterile distilled water is used for the PCR purpose. The details of the primers used were described in table 3.1.

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

Virus target	**Primer name**	**Sequence (5'-3')**	**Amplified region**	**Amplicon size**
Multiplex	ICMV_A_F	GCTGATTCTGGCA TTTGTAN	Common region and part of AC1	600 bp (SLCMV) 900 bp (ICMV)
	SLCMV_A_F	TGTAATTCTCAAA AGTTACAGTCN		
	I/SLCMV _A_R	ATATGGACCACATC GTGTCN		
Coat Protein gene	CP-F	GGA TCC ATG TCG AAG CGACCA	Coat protein gene	770 bp
	CP-R	AAG CTT TTA ATT GCT GAC CGA		

Table 3.2: Components of PCR reaction mix is listed below:

Components	**Volume**
10x buffer for Dynazyme polymerase II (Thermo scientific)	2.5 µl
dNTP (10 mM)	1 µl
ICMV-A-F (10pmol μl^{-1})	0.5 µl

SLCMV-A-F (10pmol μl^{-1})	0.5 µl
ICMV/SLCMV-A-R (10pmol μl^{-1})	0.5 µl
Taq polymerase(0.5U μl^{-1})	0.3 µl
Water	12.7 µl
Template DNA(50 ng μl^{-1})	2 µl
Total volume	20 µl

PCR was carried out in BioRad C1000 Touch Thermocycler (Germany). PCR programme was set with initial denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 45 sec, annealing at 55ºC for 30sec and extension at 72ºC for 1 min. Final extension was done at 72ºC for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under Azure gel documentation system.

3.3 COLLECTION AND PLANTING OF RESISTANT LINES FOR SCREENING

Resistant varieties were collected and planted to assess their resistance level against cassava mosaic virus infection through grafting. The selected plants include 15s 103, 15s 409, 8S 501-2, 9s 132, 17s 143, 17s 135, 17s 241, CI 889, 17s 209, 9s 75, 15s 436, 17s 48, 17s 39, CR 54 A3, 15s 278. Fifteen plants were maintained for each variety to assess their level of resistance through the grafting approach. Details regarding the breeding lines are explained in Table 3.3.

Table 3.3: Details of breeding lines used for resistance screening.

Sl. No.	**Sample name**	**Pedigree**
1	H-226	Released Variety
2	15s 103	Breeding line (Hybrid)
3	15s 409	Breeding line (Hybrid)

4	8S-501-2	Inbred
5	9s 132	Breeding line (Hybrid)
6	17s 143	Breeding line (Hybrid)
7	17s 135	Breeding line (Hybrid)
8	17s 241	Breeding line (Hybrid)
9	CI 889	Landrace
10	17s 209	Breeding line (Hybrid)
11	9s 75	Breeding line (Hybrid)
12	15s 436	Breeding line (Hybrid)
13	17s 48	Breeding line (Hybrid)
14	17s 39	Breeding line (Hybrid)
15	CR 54 A3	Exotic accession
16	15s 278	Breeding line (Hybrid)

3.4 MULTIPLICATION OF VIRUS-FREE SUSCEPTIBLE CASSAVA CULTIVAR (H-226) UNDER *IN VITRO* AND HARDENING

The CMD susceptible plants of cassava cultivar H-226 were multiplied by tissue culture. Virus- free plants of cv.H-226 established through meristem culture in the lab were used as source plant for multiplication in large number under *in vitro* conditions through micropropagation. Cassava basal medium (Appendix III) was used for the subculture of H-226. The well grown plant was selected and hardened to obtain the control plant free of viral infection. For hardening, the rooted shoots are carefully taken out from the tubes. Washed it thoroughly to remove the medium adhered to the roots of the plant. Dipped the plant in 0.1% bavistin solution and planted in pot trays filled with coir pith and soil. The trays were shifted to a mist chamber with 50% shade. After 3 weeks the plants are shifted to large pots with soil. The plant was kept in such a way as to strictly protect it from direct contact with the environment to avoid viral infection.

3.5 ISOLATION OF DNA AND DIAGNOSIS FROM RESISTANT LINES AND *IN VITRO* PLANTS

The total DNA of the 15 breeding lines and *in vitro* raised H-226 plant was isolated by CTAB method described by Lodhi *et al.* (1994). The quality of the DNA was analyzed by using Agarose gel electrophoresis and quantity was documented by spectrophotometer. The virus load was checked by using Polymerase Chain Reaction using the primers described in Table 3.1. The amplified products were viewed under Azure gel documentation system along with PCR marker.

3.6 GRAFTING OF RESISTANT AND SUSCEPTIBLE LINES AND OBSERVATION ON DISEASE DEVELOPMENT

Grafting is used to transmit viral infection to the plant which is free of virus. Chip bud grafting was carried out with the resistant lines to know its resistance against SLCMV. In this method, a single bud is attached with the rootstock to make the complete plant.

3.6.1 Selection of suitable bud

Scion used for the chip budding procedure was a cassava mosaic virus confirmed plant variety kariyilaporiyan. A suitable plant with a similar diameter of stock plant was selected for grafting. A small incision was made in the infected plant using a sterile blade at an angle of 45º. The bud was carefully removed from the infected plant along with the cambium regions. The buds were detached carefully from the infected plant without any damage and immediately kept the bud in water to avoid dehydration.

3.6.2 Chip bud grafting

The stock plants which are of resistant lines and healthy H-226 were selected for the grafting. Excised bud from infected plant was carefully fixed to the incision made on the stock plant with similar diameter. Then bud graft is secured with parafilm to avoid

contact with external environment. The callus formation was seen within 1 week. Within 2-4 weeks the new leaf was raised from the graft.

3.7 CONFIRMATION OF INFECTION OR ABSENCE OF INFECTION THROUGH MOLECULAR ANALYSIS

3.7.1 Enzyme Linked Immunosorbent Assay (ELISA)

Triple Antibody Sandwich ELISA (TAS-ELISA) for CMD detection was carried out using collected cassava leaf samples showing different symptoms of virus infection as well as resistant lines. A healthy non host sample collected from the field was used as the negative control. Wells of ELISA microtitre plates were coated with 200µl of purified IgG (ACMV Polyclonal antibody) diluted to 1:1000 in coating buffer (Appendix IV) and incubated at 37°C for 3 hrs. The plates were washed three times with PBS-T buffer (Appendix IV), soaked for three minutes during each wash and dried by tapping upside down on a tissue paper spread on the bench. When the plates became completely dried, 200µl of blocking solution (Appendix IV) was added to each well (blocking) and incubated at 37°C for 1 hr. After the incubation period, the blocking solution was removed; tap dried the plates and again washed three times using PBS-T buffer. After the plates become dried, 200µl of test samples was loaded in duplicate wells and incubated overnight at 4°C. The test samples were prepared by grinding 100 mg test leaves in sample extraction buffer (Appendix IV) and centrifuged at 8000 rpm for 10 minutes and the supernatant was taken. After the incubation period, plates were washed thrice using PBS-Tween solution and tap dried. Then the plates were coated with 200 µl of monoclonal antibody (SLCMV MAb) diluted to 1:500 in conjugate buffer (Appendix IV) and plates were incubated at 37°C for 3 hrs. After the incubation period, the plates were washed thrice using PBS- T buffer and tap dried. Then the plates were coated with 200 µl of conjugate antibody [Alkaline Phosphatase (ALP) conjugated anti-mouse IgG] in appropriate conjugate buffer (Appendix IV) incubated at 37°C for 2 hrs. After washing and drying, 200 µl aliquots of freshly prepared substrate (Appendix IV) dissolved in 10

ml of substrate buffer (Appendix IV) was added to each well and incubated at room temperature in dark condition.

The absorbance at 405nm for sample in each well was measured in a BIO-RAD iMark Microplate Reader (USA). The readings were taken at 0 hr, 30 min, 1 hr, 2 hr and overnight.

3.7.2 Polymerase Chain Reaction

The total DNA from the grafted breeding lines was isolated by CTAB method described by Lodhi *et al.* (1994). The quality of the DNA was analyzed by using Agarose gel electrophoresis and quantity was documented by spectrophotometer. Polymerase Chain Reaction was carried out in the grafted breeding lines to confirm the presence or absence of virus. The details of primers used in the reaction are described in Table 3.1. The amplified products are viewed under Azure gel documentation system along with PCR marker to check the presence or absence of virus.

3.7.3 qPCR

Working solutions of primers, DNA samples from grafted plants were stored at -20º C and not thawed more than twice prior to use. Master mixes and water were kept in small aliquots at 4ºC. 96 well micro plate is used for the reaction. For each DNA sample three replicate reactions were prepared. Micro plates were sealed immediately before performing real time PCR assay in Eppendorf realplex Mastercycler (Eppendorf, Germany).

Table 3.4: Details of primer used for the diagnosis of Cassava Mosaic virus (SLCMV) in real time PCR

Target	**Primer name**	**Sequence (5'-3')**
DNA A	Sy-SLCMV_A_F	TTCATCCATCCATATCTT

	Sy-SLCMV_A_R	CAATATAGGTAAGGTCAT
DNA B	Sy-SLCMV_B_F	AGCCATACATAATATACAAGT
	Sy-SLCMV_B_R	CCAGTTAATACGGAGAAG

Table 3.5: Components of standard RT PCR mix

Components	**Volume**
Maxima®SYBR Green qPCR master mix	12.5 µl
Forward primer	0.3 µM
Reverse primer	0.3 µM
Template DNA	≤100µg
Nuclease free water	Make up to 25 µl
Total volume	25 µl

Parameters for the cycle were set as follows: DNA polymerase activation was carried out at 95°C for 3 min. Followed by 40 cycles of denaturation at 95ºC for 15 seconds. Annealing was carried out at 53ºC for 20 seconds. The extension was done at 72ºC for 20 seconds.

4. RESULTS

The results of the study entitled “Screening of cassava genotype for cassava mosaic disease resistance.” conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2019-2020 are presented in this chapter.

4.1 COLLECTION OF INFECTED SOURCE PLANT AND ESTABLISHMENT IN GLASS HOUSE AS VIRUS INOCULAM SOURCE

Cassava mosaic virus infected samples were collected from different regions including fields near Vizhinjam as well as from CTCRI Thiruvananthapuram. Forty five leaf samples were collected from these regions according to the symptom scale represented by Hahn *et al.*, 1980. Of the collected samples six plants showed an infection scale of 1. Eight plants represented the symptom scale of 2. Ten samples showed the symptom scale of 3 and five samples were included in the symptom scale 4. Most of the plants, a total of 16 numbers were classified in the symptom scale 5. These stem cuttings of the infected plants were collected.

The collected samples were planted in the glass-house to conserve it as an inoculam source for grafting purposes (Fig. 4.1).

Fig. 4.1: Cassava mosaic diseased plants collected from fields established in the glasshouse

4.2 CONFIRMATION OF VIRUS INFECTION THROUGH PCR

The established plants in the glasshouse were tested for confirmation of virus infection. PCR method was used for the confirmation of virus infection

4.2.1 DNA isolation

Plant DNA was isolated from the infected plants by CTAB method. The quality and quantity of DNA were analyzed by Agarose gel electrophoresis and spectrophotometer respectively. The details of spectrophotometer readings are shown in Table 4.1. The image of DNA obtained by gel electrophoresis showed good quality DNA (Fig. 4.2).

Table 4.1: Spectrophotometric details of isolated DNA from the infected source plant.

Sample no.	**Concentration (ng/µl)**	**A260/280**
1	5670	1.98
2	4734	1.96
3	4038	1.82
4	5013	1.89
5	1202	1.9
6	4568	1.79
7	4376	1.83
8	6231	1.81

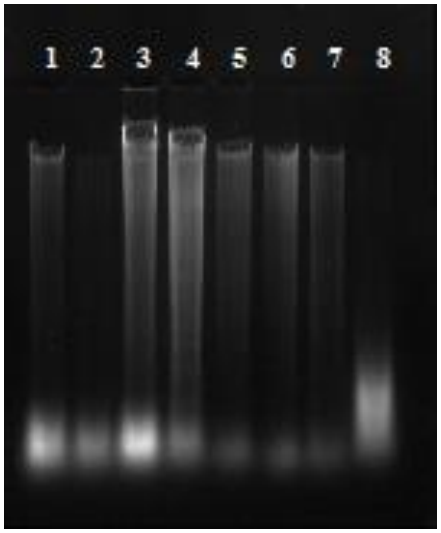

Fig. 4.2: DNA samples obtained from infected plants collected from fields. Lane 1-7 cassava mosaic infected leaf samples. Lane 8 shows the DNA of tissue culture-derived virus free-H-226.

4.2.2 Multiplex PCR

Multiplex PCR was performed for the detection of virus present in the sample. Of the samples collected one sample showed ICMV infection and the rest of them were SLCMV infected (Fig. 4.3).

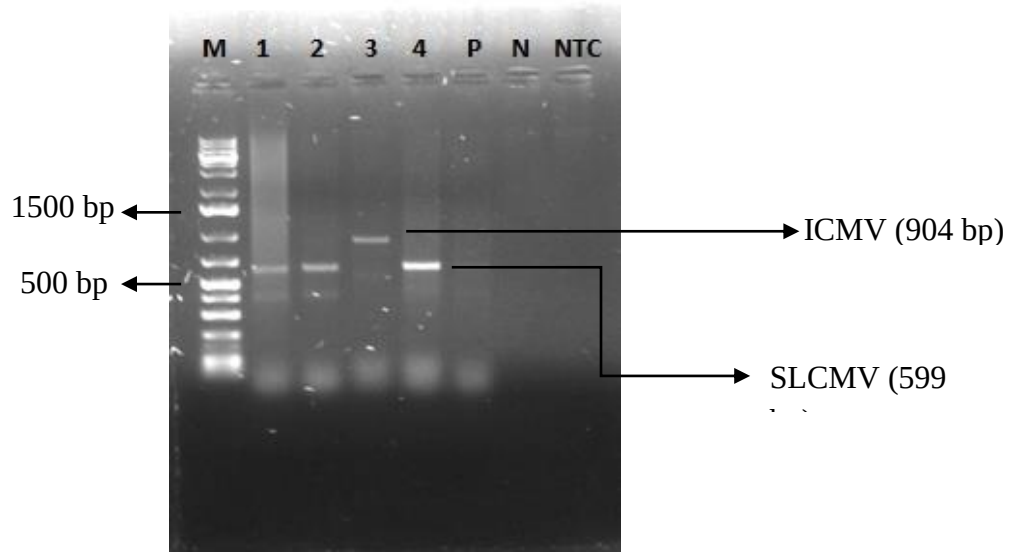

Fig. 4.3: Image showing multiplex PCR products of the plants in 1% gel. M- 1 kb plus DNA ladder (Thermo Scientific), 1, 2, 4- SLCMV positive samples, 3- ICMV positive sample, P- Positive control, N- Negative control

4.3 COLLECTION AND PLANTING OF RESISTANT LINES FOR SCREENING

For CMD resistance screening study, 15 resistance lines of cassava were collected from ICAR-CTCRI Thiruvananthapuram. They were maintained well in the glasshouse for further analysis. The details of resistant line collected for the analysis include 15S 103, 15S 409, 8S 501-2, 9S132, 17S 143, 17S 135, 17S 241, CI 889, 17S 209, 9S75, 15S436, 17S48, 17S 39, CR 54 A3, 15S 278. These 15 lines were maintained for grafting with infected buds for screening and further analysis (Fig. 4.4).

Fig. 4.4: Some of the cassava mosaic resistant breeding lines used for the study.

4.4 MULTIPLICATION OF VIRUS-FREE SUSCEPTIBLE CASSAVA CULTIVAR (cv.H-226) UNDER *IN VITRO* AND HARDENING

4.4.1 Sub culturing of cv.H-226

Virus- free cv.H-226 established through meristem culture was used for sub culturing. Multiplications of these plants in vitro were carried out using nodal segments from the *in vitro* raised virus-free plants (Fig.4.5). A large number of plants were established through micropropagation.

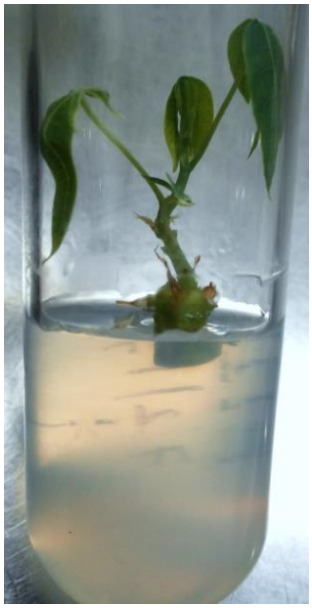

Fig. 4.5: Micropropagation of cassava cv.H-226

4.4.2 Hardening of H-226

Two weeks old sub cultured well-rooted plants are selected for hardening (Fig. 4.6). Initial hardening was done in paper cups and after two weeks of establishment they were transferred to pots (Fig.4.7).

Fig.4.6: plantlet ready for hardening

Fig. 4.7: Two weeks after hardening ofcv.H-226

4.5 MULTIPLEX PCR BASED DIAGNOSIS TO ANALYSE VIRAL INFECTION

4.5.1 Plant DNA isolation

Leaf samples of plants were analyzed by using CTAB method. DNA from both resistant lines as well as from the control leaves (*in vitro* cultured H-226) was isolated. The quality and quantity of DNA were analyzed using 0.8% agarose gel electrophoresis and spectrometer respectively. Table 4.2 shows detailed spectrophotometer readings. Analysis of the isolated DNA in agarose gel electrophoresis showed good quality (Fig. 4.8).

Table 4.2: Spectrophotometric details of isolated DNA from breeding lines.

Sl. No.	**Sample name**	**Concentration (ng/µl)**	**A260/280**
1	Control	4560	1.73
2	15s 103	4280	1.89
3	15s 409	6329	1.87
4	8S 501-2	3517	1.93
5	9s 132	1977	2.01
6	17s 143	4127	1.98
7	17s 135	4048	1.81
8	17s 241	5425	1.90
9	CI 889	3336	1.79
10	17s 209	5652	1.85
11	9s 75	3126	1.83
12	15s 436	2043	1.83
13	17s 48	7430	1.92
14	17s 39	3927	1.76
15	CR 54 A3	3255	1.81
16	15s 278	4679	193

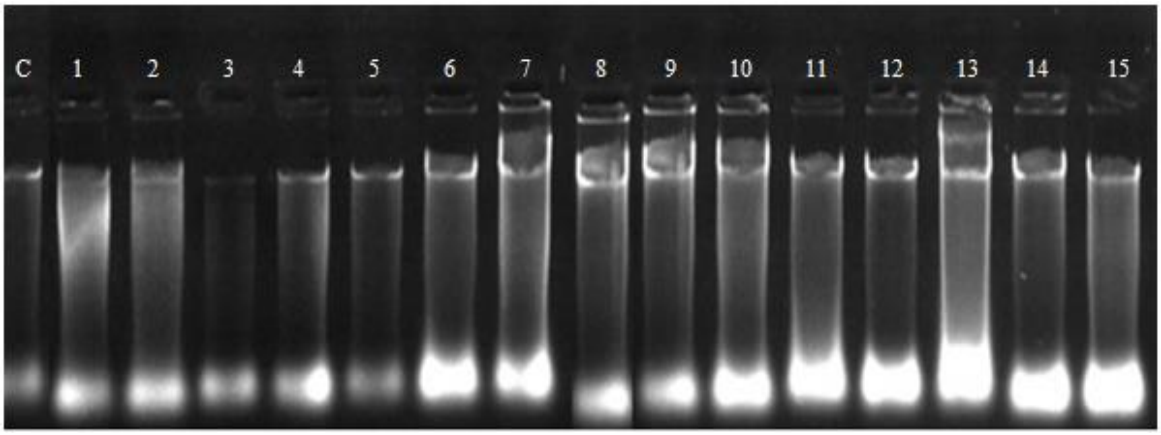

Fig.4.8: DNA samples obtained from the breeding lines and tissue culture plant. C- Shows DNA of *in vitro* cultured H-226. Lane1-15 DNA from resistant lines.

4.5.2 Polymerase Chain Reaction

The absence of ICMV or SLCMV in 15 resistant lines of cassava and *in vitro* raised H-226 was confirmed through multiplex PCR. Gel analysis of amplified products showed that all the resistant lines and meristem derived H-226 plants were free from virus infection (Fig. 4.9).

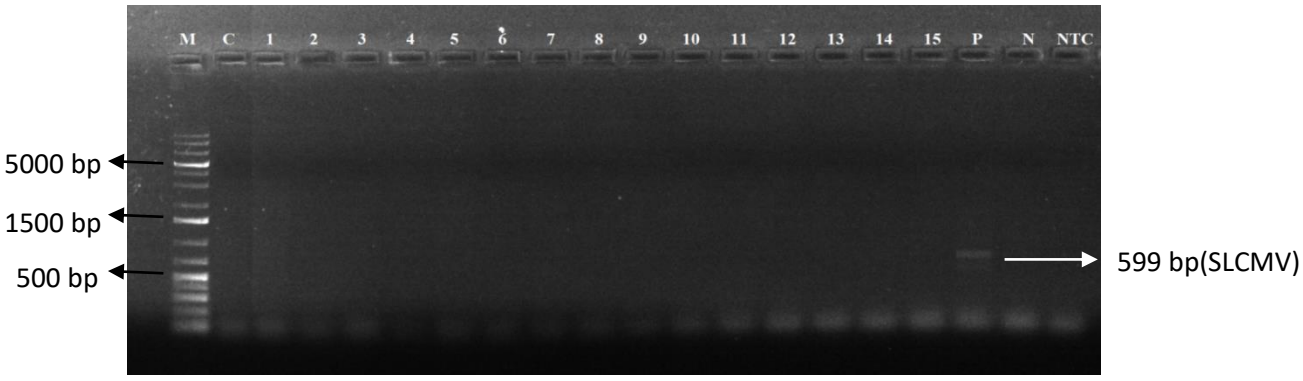

Fig. 4.9: Gel image showing multiplex PCR products of samples. C- Control plant (*in vitro* cultured H-226), 1-15 Virus free breeding lines, P- Positive control, N- Negative control

4.6 GRAFTING OF RESISTANT AND SUSCEPTIBLE LINES AND OBSERVATION ON DISEASE DEVELOPMENT

The breeding lines were selected as a stock plant and the established infected plant in the glasshouse was the scion. Fig. 4.10 shows the steps involved in the chip bud grafting.

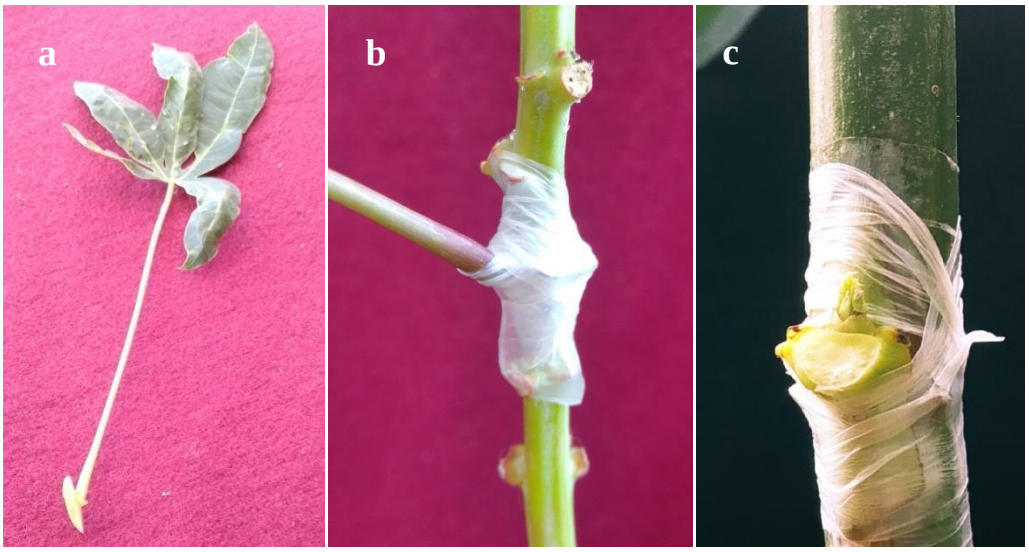

Fig. 4.10: Steps in chip bud grafting of cassava. (a) Axillary bud removed from the virus infected plant (scion) (b) Graft is secured with the parafilm (c) Emergence of bud after successful grafting

Three plants from each breeding line as well as from the control were used as the replica for the study. The graft emergence occurred at 2nd week after chip bud grafting (Fig.4.11). The upper region just above the graft emergence was carefully removed. Time taken for the emergence of plants from the graft were varied from 10 – 13 days (Table 4.3).

Table 4.3: The details of graft emergence.

No.	**Name of breeding line**	**Date of grafting (Chip bud grafting)**	**Graft emerging time**
1	9s 132	08 June 2020	21 June 2020 (13 Days)
2	15s 103	08 June 2020	20 June 2020 (12 Days)
3	17s 135	08 June 2020	20June 2020 (12 Days)
4	15s 409	08 June 2020	18 June 2020 (10 Days)
5	8S 501-2	08 June 2020	18 June 2020 (10 Days)
6	17s 241	08 June 2020	18 June 2020 (10 Days)
7	CI 889	08 June 2020	20 June 2020 (12Days)
8	17s 209	08 June 2020	19 June 2020 (11 Days)
9	9s 75	08 June 2020	20 June 2020 (12 Days)
10	15s 436	08 June 2020	18 June 2020 (10 Days)
11	17s 48	08 June 2020	19 June 2020 (11 Days)
12	17s 39	08 June 2020	21 June 2020 (13Days)
13	CR 54 A3	08 June 2020	20 June 2020 (12 Days)
14	15s 278	08 June 2020	18 June 2020 (10 Days)
15	17s 143	08 June 2020	19 June 2020 (11 Days)
16	H-226(Control plant)	08 june 2020	20 June 2020 (12 Days)


9S 132


15S 103


15S 409


17S 135

Fig. 4.11: Graft emergence in the grafted plants

After 4 weeks, well developed shoots having leaves were observed (Fig.4.12). As no symptoms were observed in the emerged leaves, leaf samples were collected from all the grafted plants for molecular analysis to know the presence or absence of virus.

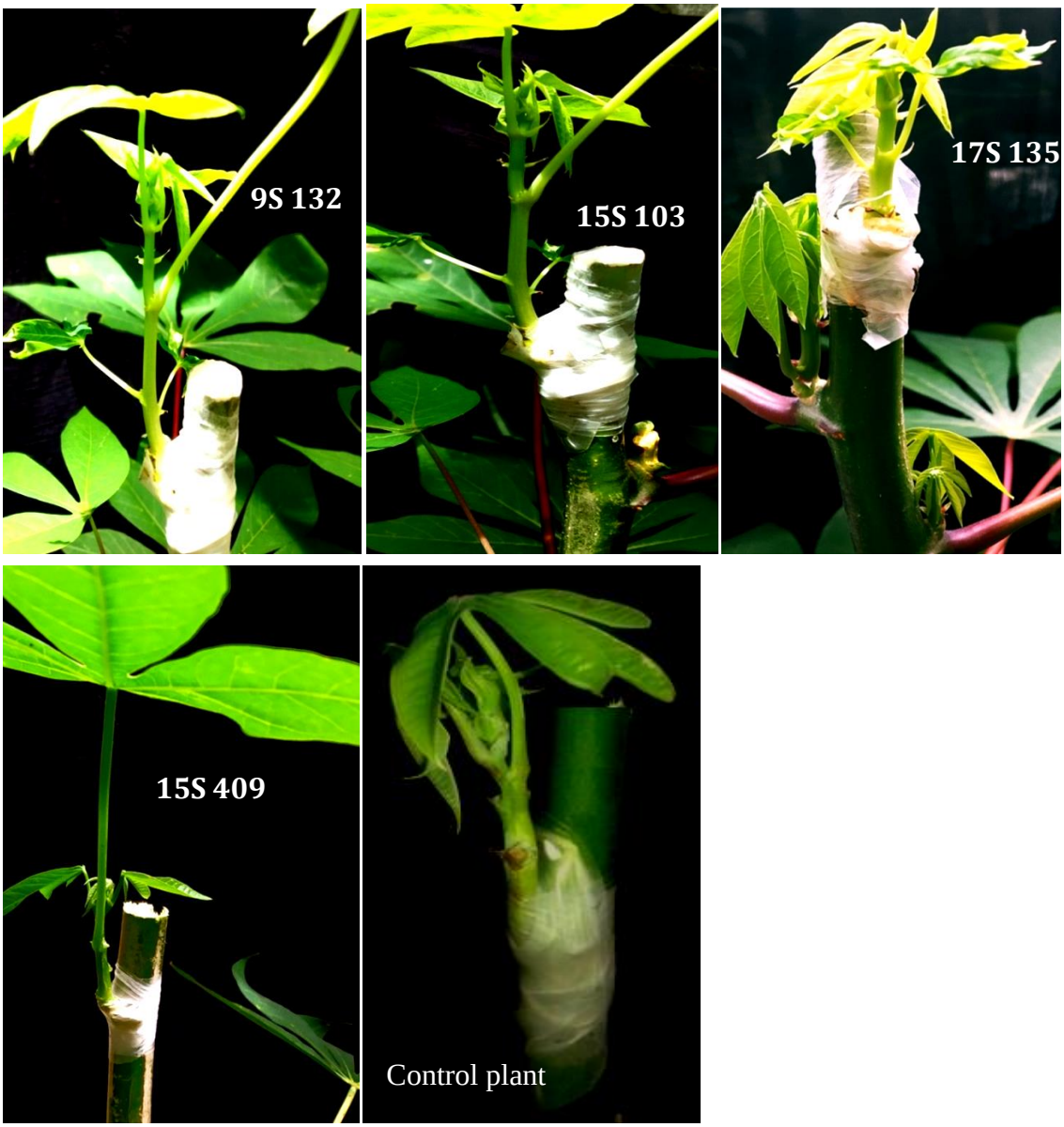

Fig. 4.12: Emergence of shoot with leaves in the grafted plants and control plant with visible symptoms.

After 2 month of grafting, the plants remain healthy and symptom free. Severe symptom was observed only in meristem derived H-226 used for grafting with infected bud (Fig.4.13). Leaves were collected from these plants for the analysis of virus load.

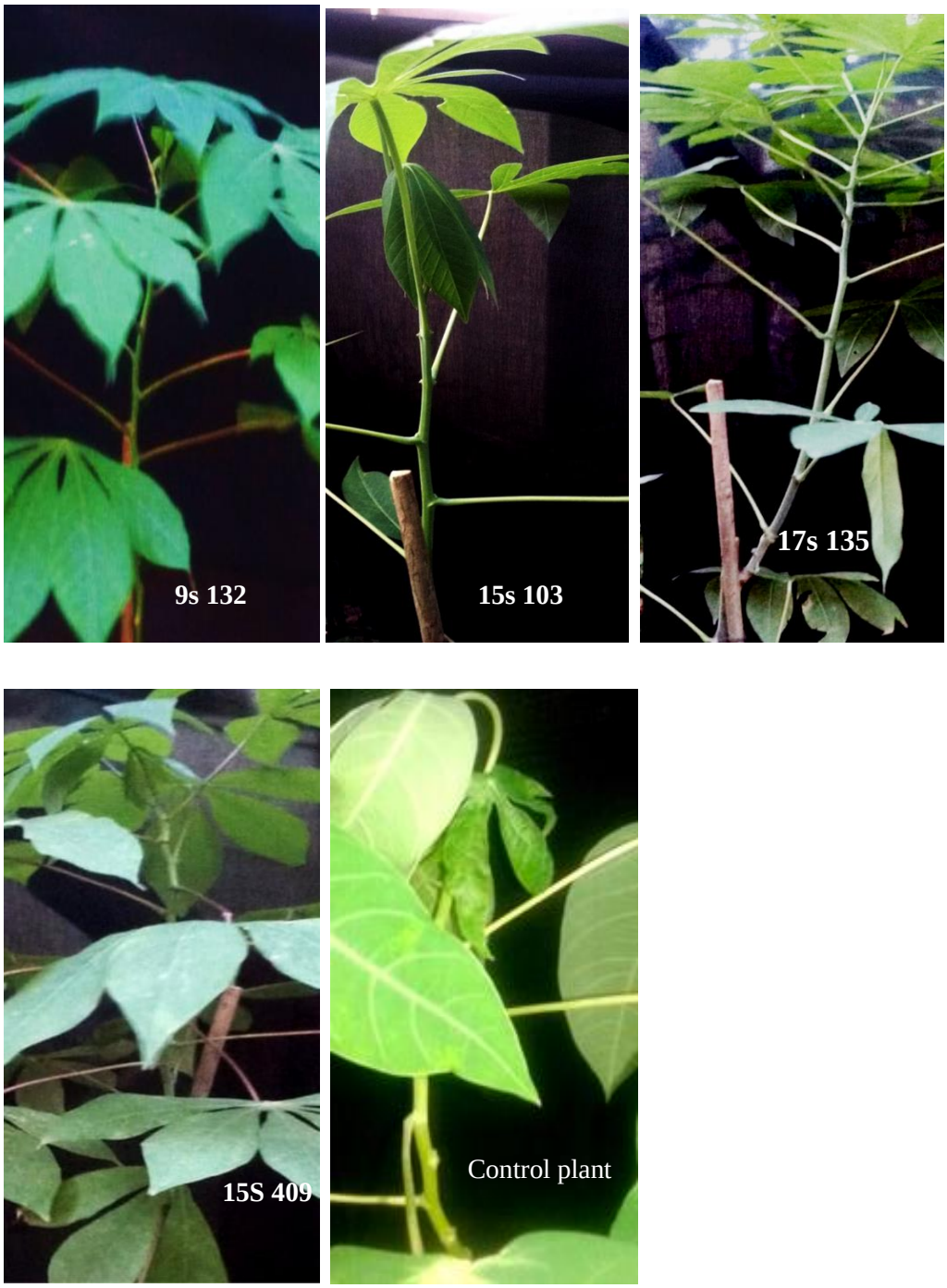

Fig. 4.13: Images of grafted plants after 2nd month of grafting.

4.7 CONFIRMATION OF INFECTION OR ABSENCE OF INFECTION THROUGH MOLECULAR ANALYSIS

4.7.1 Molecular analysis through PCR

Gel electrophoretic analysis of total DNA isolated from the grafted samples of control as well as breeding lines showed that they are all in good quality and suitable for molecular analysis (Fig. 4.14). The polymerase chain reaction (PCR) was carried out to find out the detection of presence or absence of SLCMV/ICMV in these samples using multiplex primer immediately after the first new shoot emerged from the grafted plant. Analysis of amplified products in agarose gel electrophoresis showed that amplification was observed only in susceptible line (H-226) with a band size of 600 bp for SLCMV and no bands were present in breeding lines (Fig. 4.15). This confirmed that the breeding lines are free from ICMV/SLCMV infection and they are resistant to Cassava mosaic disease.

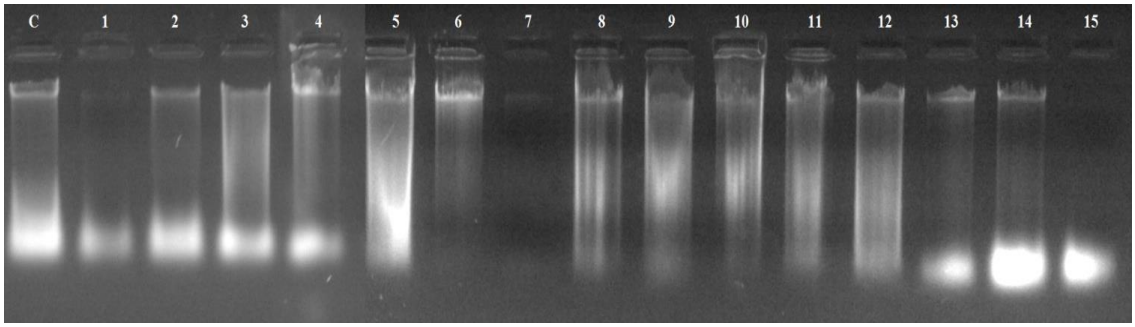

Fig. 4.14: DNA samples obtained from the grafted plants. C- Control plant (*in vitro* cultured H-226), 1-15 DNA from breeding lines.

Fig. 4.15: Gel analysis of PCR products of grafted samples. C- Control plant (*in vitro* cultured H-226), 1-15 breeding lines, P- Positive control, N- Negative control

The plants were again tested for the presence or absence of the virus after 2 months of grafting. The PCR image of analysed plants is shown in Fig. 4.16.

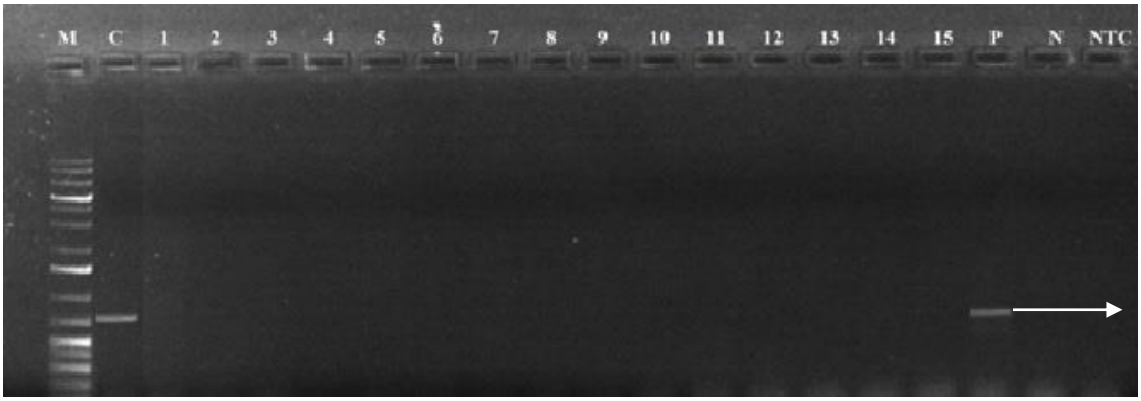

Fig.4.16: PCR based diagnosis of SLCMV in grafted cassava plants after two months of grafting.

4.7.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Triple Antibody Sandwich ELISA (TAS-ELISA) was done to analyze all the grafted plants for the presence or absence of virus infection in grafted plants. The TAS-ELISA readings (at 405 nm) of grafted samples (Table 4.4) showed that positive reaction was obtained only H-226 grafted plant and all the breeding lines showed no reaction which showed that all these lines were resistant to cassava mosaic virus infection.

Table. 4.4: TAS-ELISA results of the grafted samples.

Sample No:	**Sample Name**	**OD $_{405}$**	**Result**
1	H-226(Control)	1.917	+
2	15s 103	0.257	-

3	15s 409	0.241	-
4	8S 501-2	0.256	-
5	9s 132	0.247	-
6	17s 143	0.27	-
7	17s 135	0.33	-
8	17s 241	0.245	-
9	CI 889	0.253	-
10	17s 209	0.271	-
11	9s 75	0.24	-
12	15s 436	0.217	-
13	17s 48	0.233	-
14	17s 39	0.293	-
15	CR54A3	0.223	-
16	15s 278	0.241	-
17	Positive	1.013	+
18	Negative	0.182	-
19	Buffer control	0.067	

4.7.3 qPCR

Quantitative PCR was carried out to analyze the presence or absence of the virus. The virus load was assigned both in breeding lines as well as in the control plant. The detail regarding the Real-time PCR analysis is detailed in table 4.5. Ct mean value, as well as number of copies (virus load), was observed higher only for control plant (H-226) which showed symptoms and all the breeding lines grafted with infected buds had very low Ct mean value and very few copies of virus load which showed a negative reaction. Now it revealed that all the breeding lines screened in the present study are resistant to *Sri Lankan Cassava Mosaic Virus.*

Table. 4.5: q-PCR results of the grafted samples.

Sample No:	**Sample Name**	**Ct Mean**	**Number of copies (Virus load)**
1	H-226 (Control)	1.05	2.36×10^{13}
2	15s 103	0.04	207
3	15s 409	0.13	734
4	8S 501-2	0.03	186
5	9s 132	0.07	432
6	17s 143	0.13	856
7	17s 135	0.11	973
8	17s 241	0.1	508
9	CI 889	0.24	1086
10	17s 209	0.01	79
11	9s 75	0.13	739
12	15s 436	0.09	587
13	17s 48	0.11	649
14	17s 39	0.08	482
15	CR54A3	0.2	953
16	15s 278	0.16	594
17	Positive	1.67	1.57×10^{69}
18	Negative	-	
19	NTC	-	

5. DISCUSSION

Cassava (*Manihot esculenta* subspecies *esculenta* Crantz) is a perennial shrub belonging to the family Euphorbiaceae, an important vegetatively propagated food crop in the tropics. They are being adapted to large climatic conditions and are the major source of dietary starch along with vitamins, fiber, minerals and proteins. Cassava is vulnerable to many diseases caused by viruses; within this cassava mosaic disease (CMD) is the most important disease which limits the production of cassava. Cassava mosaic begomovirus (CMB) (genus *Begomovirus*, family Geminiviridae) is the causative agent for CMD and the spread of the disease is mainly through infectious stem cuttings and whitefly vector. The development of cassava mosaic disease-resistant lines is the ultimate solution for the control of disease rather than depending on other methods.

Eleven species of cassava mosaic Gemini-virus species have been identified in association with cassava mosaic disease. Warburg (1984) reported the presence of *African cassava Mosaic Virus* for the first time in Tanzania. Malathi *et al.* (1985) first reported the presence of *Indian cassava mosaic virus* from South Asia. Saunders *et al.*, (2002) recorded *Sri Lankan cassava mosaic virus*, another virus which is flourished widely in Asia. Cassava Mosaic Disease (CMD) is widely spread in Africa and Asia, and is associated with mixed virus infections and recombinant and re-assorted virus strains (Siriwan *et al.*, 2020).

The characteristics of cassava mosaic disease include distortion, stunted leaf and sometimes the entire plant will be affected. It affects severe yield loss in cassava production in all over the world (Lamptey *et al.*, 2000). The virus can be transmitted more easily by infected stem cutting and the spreading of virus is highly linked with the vector *Bemisia tabaci* (Fargette and Fauquet, 1988).

The genomic component of cassava mosaic geminiviruses formed of DNA A and DNA B (circular ssDNA molecule) which is of about 2.8kb each. The genome replication is by means of rolling circle amplification through a dsDNA intermediate stage (Hanley

et al., 1999). AC1 to AC4 (complimentary sense) are the four genes that encode DNA A and AV1, AV2 are the two in the sense which is responsible for replication, transcription enhancement and encapsulation. DNA-B encodes one gene in the virion *BV1* and *BC1* (complementary senses) respectively, required for intra and intercellular movement (Jeske *et al.,* 2001).

Molecular detection of viruses can be analyzed by means of several serological methods and with most reliable and prominent PCR based methods. Virus-specific primers could analyze the infection as well as could differentiate the virus. For detection of *Indian Cassava Mosaic Virus* primers specific to full length coat protein (CP), Replicase (AC1), movement protein (MP) and partial coat protein (CP1) primers were designed (Makeshkumar *et al.,* 2005). ELISA, DIBA, TBIA are certain serological approaches used for the detection of ICMV (Malathi *et al.,* 1985; Malathi *et al.,* 1989; Makeshkumar and Nair, 2001).

Phytosanitation is the well-adapted effective method for controlling the spread of cassava mosaic disease. Through this method, the disease-free surviving plants are used for the propagation by removing diseased plants within the plantings. The virus-free plants not infected until the last stage of crop growth are selected for propagation (Thresh, 2006). Spraying of pesticides to reduce the population of whitefly vector will help in preventing the spread of the disease to a certain extent. Disease resistance varieties identified for cassava mosaic disease with desirable features could effectively introduce for better results.

The most effective management of controlling the spreading of CMD is by introducing improved plants with CMD resistance. Breeding the crop tolerant to the virus can be done by either genetic engineering or by breeding approaches (Lapidot and Friedmann, 2002). Interspecific hybridization by the integration of resistant trait from *Manihot glaziovii* developed CMD resistant cassava in Africa, which has become the major source of dominating CMD resistance (Fargette *et al.,* 1996).

One of the most important breeding objectives of cassava is to enhancing resistance to cassava mosaic disease to reduce the yield loss. Now the resistant breeding is on good progress but still there is need for increasing the resistance levels to the gene pool. Lokko *et al.* (2006) conducted resistance analysis to know the combining ability analysis of resistance to mosaic disease in cassava. The breeding lines were tested by artificial inoculation methods like bud grafting. Laboratory based methods like biolistic delivery of cloned virus and RNA silencing mechanisms.

Resistance evaluation for CMD resistance was done by Hounge *et al.* (2018) in selected African cassava cultivars by using combined molecular and greenhouse grafting tools. Molecular screening of grafted cultivars showed molecular markers associated with cmd 2 gene revealed lower CMD symptoms. But the cultivar BEN 86052 was also showed reduced symptoms with the absence of the gene CMD 2. It showed that genetic background of the plants has several roles in maintaining resistance. Three resistance genes, *CMD1* (recessive), *CMD2* (dominant) and *CMD3* (QTL conferring resistance) have been used in molecular CMD resistance screening of cassava, with CMD2 being the most widely used (Beyene *et al.*, 2016).

The rapid screening system for determining resistance and susceptibility to CMD is done based on virus-induced gene silencing (VIGS) of an endogenous cassava gene. The method is space and resource-efficient, reducing the time required to perform CMD screening to two to four weeks. It can be employed as a high throughput rapid screening system to assess new cassava cultivars and for screening transgenic, gene-edited and breeding lines under controlled growth conditions (Beyene *et al.*, 2017).

RNA silencing is the most important method established in plants as an adaptive defense mechanism. Virus derived siRNA implicating virus-induced post-transcriptional gene silencing (PTGS) have effectively control the virus to develop disease free-plants. (Chellappan *et al.*, 2004).

Biolistic inoculation of cassava mosaic begomoviruses of the screening for resistance to Cassava mosaic was studied by Ariyo *et al.* (2003). Infectious clones of cassava mosaic Geminiviruses were used for the virus inoculation by using gene gun facility. DNA extracts from the infected plants and cloned viruses were used for the coating of gold particle. The study done with *East African cassava mosaic viruses* could able to verify 96/0160 was resistant to the virus attack. AFLP is another approach used in African Cassava germplasm for the resistance analysis to the CMD. Amplified Fragment Length Polymorphism was assessed in the 20 landraces and 9 elite lines of cassava were used in the analysis. The unique AFLP Fragment present in the African accessions could able to understand the process of genetic divergence with the Latin American accessions (Fregene *et al.*, 2000).

Monde *et al.* (2012) carried out resistance screening by grafting and whitefly inoculation in local and improved cassava. The whiteflies were introduced to the testing lines for symptom development. Detopping of young leaves induced triggered effect on the CMD expression. EACMV- UG's showed preferential transmission by means of whitefly which was analyzed by PCR and ELISA. TMSI 95/0211, TMS 92/297, TMS 91/377 and 99/0038 were confirmed for CMD resistance.

The present study focused on the screening of developed breeding lines of cassava for resistance analysis through grafting. The infectious plants were used as the scion for grafting which was earlier confirmed by means of polymerase chain reaction. Chip bud grafting (Wagaba *et al*., 2013) was used as the methodology for resistance screening within the breeding lines. All the 15 breeding lines screened in the present study showed complete resistance against *Sri Lankan cassava mosaic virus*.

6. SUMMARY

A study on “Screening of cassava genotype against cassava mosaic disease resistance” was conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2019- 2020. The important findings of the above studies are summarised in this chapter.

For the identification of infected source plants, forty-five leaves samples were collected according to the symptom scale. From the collected samples only one sample was found infected with ICMV and the rest were tested positive for SLCMV by multiplex PCR analysis.

Fifteen cassava breeding lines developed in the breeding programme were collected from the fields of CTCRI, Sreekariyam, and the absence of viruses (ICMV and SLMV) in these breeding lines was confirmed through multiplex PCR.

The virus-free CMD susceptible cassava variety H-226 was developed through meristem culture and multiplied through micro propagation. The hardened susceptible virus-free plant maintained in the insect-proof glasshouse served as the control for the resistance screening programme. The absence of virus in these plants was also confirmed by frequent testing through PCR.

The most inexpensive as well as the most efficient “chip bud grafting” method was used for the resistance screening analysis. The stock portion was served by breeding line (negative for viral infection) and already established infected plants (positive to viral infection) were selected as the scion for the experiment. Successful grafting was established in the breeding lines as well as in the control plant.

After the second month of successful graft establishment, the newly emerged leaves were taken from the plant and tested for the presence or absence of the virus. PCR analysis using specific primers for the coat protein (CP) gene of SLCMV showed that all the plants were free of viral infection except for the control plant.

TAS-ELISA with SLCMV specific monoclonal antibody was used to diagnose the samples screened for CMD resistance by grafting. A positive reaction was obtained for control plants and all breeding lines were tested negative.

The resistance screening by chip bud grafting with combined molecular analyses by means of PCR, ELISA and q PCR confirmed that the breeding lines 15S 103, 15S 409, 8S 501-2, 9S 132, 17S 143, 17S 135, 17S 241, CI 889, 17S 209, 9S 75, 15S 436, 17S 48, 17S 39, CR 54 A3, 15S 278 are resistance to cassava mosaic virus infection.

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8. APPENDICES

APPENDIX I

Reagents required for plant total DNA isolation

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

Chloroform-Isoamyl alcohol (24: 1)

Chloroform	: 24 ml
Isoamyl alcohol	: 1 ml

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 mlGel loading dye

APPENDIX III

Cassava basal medium

Murashige and skoog medium (Including Vitamins) : 4.4g/L

Sucrose : 20g/L

CuSO4 (2mM) : 1ml/L

Adjust the pH to5.8with 1N NaOH

Makeup to 1L

Agar : 6.8 g/L

APPENDIX IV

ELISA

Reagents required for ELISA

Coating Buffer

Na_2CO_3 : 1.59 g

$NaHCO_3$: 2.93 g

NaN_3 : 0.20 g

Adjust pH to 9.6 with 1 N NaOH and make up to 1L. Autoclave and store at 4°C

Phosphate Buffered Saline (PBS) (pH 7.4)

NaCl : 8.0 g

Na_2HPO_4. $12H_2O$ (or 1.16 g Na_2HPO_4) : 2.9 g

KCl : 0.2 g

KH_2PO_4 : 0.2 g

Dissolve in 1L distilled water and check pH of the final solution

PBS-Tween (PBST)

Add 0.5 ml of Tween-20 to1 L PBS.

Sample Extraction Buffer

PBS-T + 2% PVP

Conjugate Buffer

PBS-T + 2% PVP + 0.2% egg albumin

Substrate Buffer

Diethanolamine : 9.7 ml

Distilled Water : 80 ml

Adjust the pH to 9.8 with 1 M HCl and make up to 100ml

Autoclave and store at room temperature

Substrate for Alkaline phosphatase (freshly prepared)

Dissolve 0.5 mg/ml PNPP in substrate buffer

Blocking Buffer

PBS-T+ 2% spray dried milk

9. ABSTRACT

The study on “Screening of cassava genotype for cassava mosaic disease resistance” was conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2019-2020.

The objective of the study was to screen the cassava breeding lines for resistance to cassava mosaic disease. The screening was carried out in fifteen selected cassava breeding lines from the breeding programme to analyze their resistance to cassava mosaic disease (CMD).

For the analysis of resistance, virus inoculum was introduced into the fifteen breeding lines by grafting. Chip bud grafting method was carried out to analyze the resistance of breeding lines. The *Sri Lankan cassava mosaic virus* (SLCMV) infected plants, confirmed through multiplex PCR were selected as the inoculum source (scion) for grafting.

Periodic observations were carried out in grafted plants by morphological (symptom appearance) as well as molecular analysis by means of ELISA and PCR. None of the grafted cassava plants showed any mosaic symptoms in the new shoots developed. PCR, ELISA and qPCR results revealed that the presence of SLCMV was only confirmed to the control plants (susceptible cv. H-226) and were absent in the breeding lines tested. This confirmed the 15 breeding lines screened are resistant to cassava mosaic disease caused by SLCMV.

CMD resistant cassava varieties have an effective role in minimizing the damage caused by the virus. The success of grafting suggests this technique as the most efficient one for virus transmission and the resistant screening programme. The rapid selection of CMD resistance lines can be offered by this technique.

The CMD resistance breeding lines have a better role in on-farm production as well as in the breeding programme.