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STANDARDIZATION OF VIRUS INOCULATION METHOD FOR CASSAVA MOSAIC DISEASE

by

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THESIS

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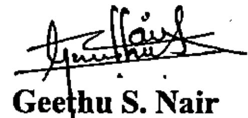
2016

DECLARATION

I hereby declare that the thesis entitled "**STANDARDIZATION OF VIRUS INOCULATION METHOD FOR CASSAVA MOSAIC DISEASE**" is a bonafide record of research done by me and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or society.



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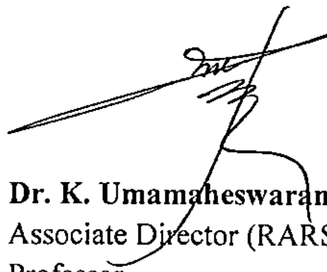

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GEETHU S. NAIR

Dedicated to my family

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LIST OF ABBREVATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
Bp	Base pair
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxy Nucleotide Tri Phosphates
EDTA	Ethylene Diamine Tetra Acetic Acid
G	Gram
hrs.	Hours
min	Minute
M	Molar
Ml	Millilitre
mM	Millimolar
NaCl	Sodium chloride
Nm	Nanometre
°C	Degree Celsius

OD	Optical Density
PCR	Polymerase Chain Reaction
rpm	Revolution per minute
S	Second
SLCMV	*Sri Lankan cassava mosaic virus*
ICMV	*Indian cassava mosaic virus*
CMD	Cassava mosaic disease
MKU	Madurai Kamaraj University
Rif	Rifampicin
Spec	Spectinomycin
kan	Kanamycin
CP	Coat protein
MP	Movement protein
IAPs	Inoculation Access Period
RCA	Rolling Circle Amplification

Introduction

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) popularly known as tapioca is an important staple food and an industrial crop, cultivated in 102 countries, from South America (Abraham, 1956). Many reports say that it is introduced into sub-Saharan Africa by the Portuguese mongers (Fauquet and Fargette, 1990), is a major food source of nearly 800 million people in tropical developing countries.

Cassava is a perennial woody shrub, which grows in tropical areas of the world. In the developing countries cassava plays an important role in agriculture because cassava grows very well in low nutrient soils with low rainfall.

In India, cassava has been in cultivation since 1960s and is majorly grown in southern India in the states of Kerala, Andhra Pradesh and Tamil Nadu. Cassava is mainly cultivated in an entire area of 0.22 million hectares with a total production of 8.1 million tonnes (FAOSTAT, 2014)

Like other crops, cassava is vulnerable to pests and diseases that can cause hefty yield losses. In cassava, more than 20 different types of viruses cause diseases (Thresh *et al.*, 1994).The major constraints in cassava production is cassava mosaic disease (CMD) and this was first recorded in India by Abraham (1956) and later by Alagianagalingam and Ramakrishnan (1966). This disease is caused by cassava mosaic virus.

Geminiviruses are an important group of plant virus, which characterized by its geminate shaped particle. These viruses are single stranded DNA with 2.7 – 2.8 kb genome (Jeske *et al.*, 2001; Hanley-Bowdoin *et al.*, 1999). Cassava mosaic geminiviruses (CMGs) belong to the genus *Begomovirus* which contains dicotyledonous plant-infecting geminiviruses (Fauquet *et al.*, 2008). The viruses have two genomic subunits, each have different genes. DNA A sub unit code for genes

responsible for replication, transcription and encapsidation, whereas DNA B sub unit codes for the genes responsible for inter and intracellular (Hull, 2002).

Globally there are 11 recognized species of Cassava mosaic geminiviruses, of which nine are reported from Africa and two species from the Indian sub-continent: *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) (Legg *et al.*,2015;Fauquet*et al.*, 2008; Patil and Fauquet, 2009). Many studies have reported that SLCMV evolved from a monopartite *begomovirus* to become a bipartite *begomovirus* by capturing the DNA-B component from ICMV (Saunders *et al.*, 2002).

Advancement in cassava breeding for virus resistance is obstruct because CMD resistance screening is tedious, and depend on natural infection conditions such as virus types at a given location and time. Therefore, developing an efficient inoculation technique with defined viruses at an early stage in breeding for resistance would provide a major improvement to the resistance development in cassava.

The present study is focused on standardization of virus inoculation method for efficient transmission of cassava mosaic virus in cassava.

Review of literature

2. REVIEW OF LITERATURE

2.1 CASSAVA

Cassava (*Manihot esculenta* Crantz) is a dicotyledonous root crop of the botanical family *Euphorbiaceae* (Fig. 1) and continues to be a food security crop for the people in the developing countries. This food crop has the capacity to withstand abiotic and biotic stresses and production per unit area.

Cassava is suited to warm humid lowland tropics where the mean annual temperature exceeds 20°C with annual rainfall that varies between 500mm and 8000 mm (Pounti-Kearlas, 1998). Although cassava tolerates drought and it grows well at rainfalls exceeding 1200mm on different types of soil. Furthermore the roots can be left in the ground for a long time before harvesting, thus giving farmers a useful security against famine. These characteristics make cassava the most cheaply cultivated crop as compared to other main staple crops such as rice, maize, wheat, and sugarcane, thus making it convenient for small-scale farmers in many tropical countries.

In India, cassava was cultivated from more than a century. In India it was introduced by the Portuguese from Brazil. Now it is cultivated in about thirteen states with major production in the states of Tamil Nadu and Kerala. The crop integrated very well with the traditions and culture of the people of south Indian states. Adaptability to poor soils, an ability to establish in high and low rainfall areas, and relative resistance to pest and disease are a few factors that greatly support the growth of cassava in these regions. In India cassava is cultivated in 2.28 lakh hectare with a production 81.39 lakh tones (FAOSTAT, 2014).

Fig. 1 Cassava (*Manihot esculenta* Crantz)

2.2 NUTRITIONAL VALUE OF CASSAVA

Cassava, a starch tuber crop, under the family *Euphorbiaceae* of plants, is the most popular edible root-vegetables. Together with other tropical roots and starch-rich foods like taro, yam, potato, plantains, etc, cassava also an essential part of carbohydrate diet for millions of peoples in the developing countries.

The calorie value of cassava is maximum than any other tropical starch rich tubers and it has nearly twice the calories than that of potatoes. Hundred grams of root provides 160 calories. Cassava is very little in fats and protein than in cereals and pulses. Vitamin-K and dietary proteins are rich in young tender cassava leaves, which has an important role in bone mass building by boost osteotrophic activity in the bones. Cassava is a balanced source of some of the B-complex group of vitamins such as thiamin, folates, riboflavin, pantothenic acid and pyridoxine (vitamin B-6). It is one of the chief sources of some important minerals like magnesium, zinc, manganese, iron and copper. In addition, it has sufficient amounts of potassium (271 mg per 100g or 6% of RDA). Potassium is an essential component of cell and body fluids that help regulate blood pressure and heart rate (FAO, 2013).

2.3 DISEASES IN CASSAVA

Cassava is vulnerable to many pests and diseases that can cause heavy yield losses. Bacteria, fungi and viruses affect the cassava crop. Main bacterial disease which affect cassava is bacterial blight (*Xanthomonas campestris* pv *manihotis*), bacterial angular leaf spot (*Xanthomonas campestris*) bacterial stem gall (*Agrobacterium tumefaciens*), Bacterial stem rot (*Erwinia carotovora*), bacterial wilt (*Erwinia herbicola*) etc. The important fungal diseases are anthracnose, black root and stem rot, blight leaf spot, brown leaf spot and cassava ash (*Oidium manihotis* Henn). Many types of viruses also affect cassava and most important among are cassava mosaic disease, cassava brown streak, cassava green mottle

2.4 CASSAVA MOSAIC DISEASE (CMD)

Cassava mosaic disease is the most important viral disease which infects cassava. The primary report of cassava mosaic disease in the Indian subcontinent was referenced by Abraham (1956) and Alagianagalingam and Ramakrishnan (1966) elaborated the CMD. *Sri Lankan cassava mosaic virus* (SLCMV), which causes a similar disease in Sri Lanka, was first documented by Austin (1986). The first clone of *Indian cassava mosaic virus* (ICMV) was obtained by Hong *et al.* (1993) and, later by Saunders *et al.* (2002) cloned SLCMV. A survey conducted in Tamil Nadu revealed that cassava mosaic disease incidence was more than 90% and the disease severity ranged from 2.35 to 4 with an overall mean of 3 ((Manivasagam *et al.,* 2006). In India ICMV caused a yield loss of 18-25% due to ICMV in India (Malathi *et al.*, 1985; Anitha *et al.,* 2011).

2.4.1 Cassava mosaic virus

Cassava mosaic geminiviruses (CMGs) is the causative agent of cassava mosaic disease, a major disease of cassava, in the African and the Indian sub-continent (Fauquet *et al.*, 2008; Patil and Fauquet, 2009; Patil *et al.*, 2005; Anitha *et al.,* 2011). The genome of each of the viruses consists of two sub genomic components, DNA-A and DNA-B, each of about 2.8 kb, with different roles in the infection process (Fig. 2). Both of the genomic components have a highly conserved intergenic common region (CR) encompassing a stem-loop structure with an invariant nonanucleotide sequence (TAATATTAC) DNA-A encodes genes responsible for viral replication [AC1 (Rep), and AC3 (Ren)], regulation of gene expression [AC2 (Trap)] and particle encapsidation [AV1 (CP)] while DNA-B encodes two proteins, BC1 (MP) and BV1 (NSP) involved in cell-to-cell movement within the plant, host range and symptom modulation.

Globally there are 11 recognized species of Cassava mosaic geminiviruses, of which nine are reported from Africa such as, *South African cassava mosaic*

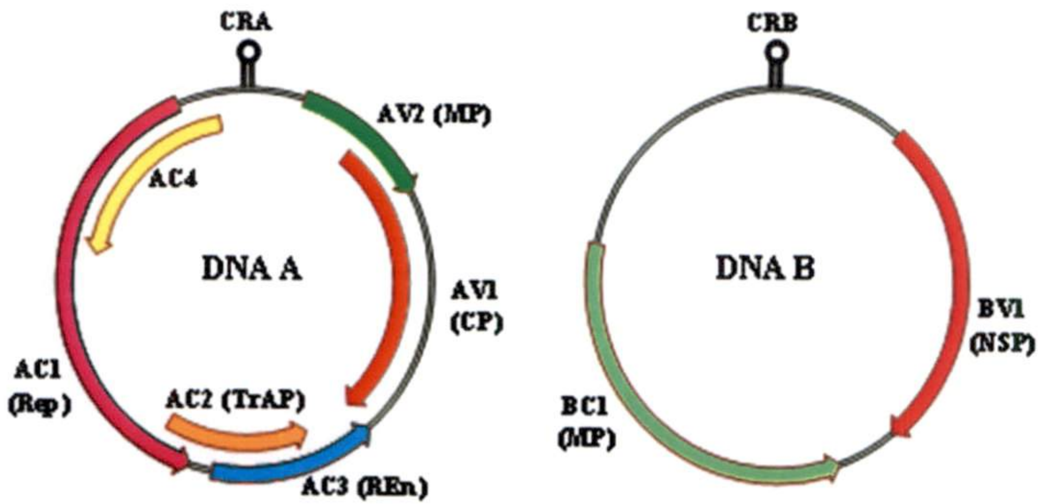


Fig. 2. Schematic representation of genome organization of cassava-infecting *begomoviruses* DNA A and DNA B components. CRA, common region A; CRB, common region B; CP, coat protein; MP, movement protein; Rep,replication-associated protein; TrAP, transcriptional activator protein; REn, replication enhancer protein.

virusAfrican cassava mosaic Burkina Faso virus, *East African cassava mosaic Kenya virus*, *Cassava mosaic Madagascar virus*, *East African cassava mosaic virus*, *East African cassava mosaic Malawi virus*, *East African cassava mosaic Zanzibar virus*, *East African cassava mosaic Cameroon virus* and *African cassava mosaic virus* and two species from the Indian sub-continent: *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) (Fauquet *et al*., 2008; Legg *et al*.,2015;Patil and Fauquet, 2009). Many studies have shown that SLCMV evolved from a monopartite *begomovirus* to become a bipartite *begomovirus* by capturing the DNA-B component from ICMV(Saunders *et al*., 2002).

2.4.2 Symptomatology

Cassava mosaic disease symptoms were first fully described by Storey and Nichols (1938). CMD symptoms can be determined at early stage of leaf development and leaf chlorosis is the primary effect of CMD, which vary from light yellow or almost white with only a little shade of green (Fig. 3). The secondary effect of CMD includes reduction in leaflet size, distortion and growth stunting. CMD symptoms varied with different factors such as difference in virus strain, age of plant and climatic conditions.

The severity of disease symptoms was assessed using the 1 to 5 scale (Hahn *et al*., 1980) that indicates the extent of symptom development (Table 1):

Table 1: The cassava mosaic symptom scale (Hahn *et al.*, 1980)

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

Fig. 3 Cassava mosaic disease symptoms

2.5 DISEASE SPREAD

CMDs are disseminated through the infected stem cuttings used for vegetative propagation. The secondary spread mainly appears in the field through the whitefly, *Bemisia tabaci* Gennadius (Fig. 4). Dissemination through the infected stem cuttings can cause the introduction of CMD to new fields where there is no spread of CMD by the whiteflies (Chant, 1958; Dubern, 1994), which is a fateful consequence of the vegetative propagation of cassava (Fargette *et al.*, 1994).

Experimental transmission can be done by biolistic inoculation by a gene gun (Briddon *et al.*, 1998). Mainly these methods have been used in resistance breeding programs for screening the genotypes against. Mechanical inoculation to *Nicotiana benthamiana* will differentiate the infection of SLCMV or ICMV. In the case of SLCMV infection, severe stunting, leaf distortion, reduction in leaf size and crinkling are commonly observed, but ICMV infection exhibits mild mottling and leaf crinkling (Makeshkumar *et al.*, 2009; Anitha *et al.*, 2011).

2.5.1 *Bemisia tabaci*

B. tabaci is indigenous to tropical and sub-tropical areas of the world. *Bemisia tabaci* belongs to the order Homoptera, family *Aleyrodidae* and is primarily a polyphagous insect that primarily colonizes annual herbaceous plants (Brown *et al.*, 1995). The abdomen lacks cornicles and the hind wings are nearly as long as the forewings (Bellows *et al.*, 1994). Most homopterans undergo gradual metamorphosis however the metamorphosis of whiteflies is different, showing a pattern more towards complete metamorphosis (Borror *et al.*, 1989). According to Borror *et al.*, (1989) there are five instars in the development cycle of *B. tabaci* including the adult. The life span of *B. tabaci* is highly depends on climatic conditions, mainly temperature (Fig. 5) (Fishpool and Burban, 1994).

Fig. 4 whitefly (*Bemisia tabaci*)

Fig. 5 *Bemisia tabaci* life cycle

B. tabaci causes many damages to crops mainly through phloem feeding. The development of insecticide resistance, monocultural practices and reduction in natural enemies have been considered as the main reasons in the emergence of *B. tabaci* as the primary agricultural pest in tropical and subtropical agricultural systems (Brown *et al.*, 1995).

The secondary spread of CMD is mainly by the vector, *B. tabaci* (Dubern, 1994; Chant, 1958) although another species of whitefly, such as *B. afer*, can also transmit cassava mosaic disease (Palaniswami *et al.*, 1996).

Heinze (1959) reported the following species of *Bemisia* as vectors of plant viruses. *B. fascialis* Jacq., *B. goldibgi* Corb., *B. inconspicua* Quaint (Quaint and Baker), *B. manihotis* Frappa, *B. nigeriensis* Corb., *B. rhodesiaensis* Corb., *B. tabaci* Gennadius, *B. tuberculata* Bondar, *B. vayssirei* Frappa, *Trialeurodes abutiloneus* (Hald.), *T. vaporariorm* , *T.natalensis* Corb., *Aleurotrachelus socialis* Bondar, *Aleurothrixus floccosus*. Of these members, the *B. tabaci* is by far the most significant vector of plant viruses (Bird and Maramorosch, 1978; Muniyappa, 1980; Bird, 1981). Alagianagalingam and Ramakrishnan (1966) reported that *Bemisia sp.* might be the vector of cassava mosaic in India. Host plant nature is an important factor which affects the puparia and putative species groups of the *B. tabaci* species complex (Thomas *et al.*, 2014).

Ellango *et al.* (2015) studied the genetic variation of *B. tabaci* population in India. Its populations differ biologically with respect to insecticide resistance, virus transmission and host range. The mtCOI- gene sequences have been useful in distinguishing genotype clusters of *B. tabaci* mainly based on geographical boundaries. They sequenced the mitochondrial *COI* (*mtCOI*) gene from *B. tabaci* populations surveyed across India. *mtCOI* sequence analyses showed the presence of Asia I, Asia I-India, Asia II-1, Asia II-5, Asia II-7, Asia II-8, and Asia II-11 genetic groups.

2.5.2 Whitefly transmission

Whiteflies *Bemisia tabaci* can be involved in direct feeding and transmission of plant viruses (Perrings, 2001).Positive correlations have been observed between *B. tabaci* populations and CMD spread into initially healthy cassava plantings (Fargette *et al.*, 1993; Legg and Raya, 1998). The size of the whitefly populations has also been positively correlated with virus spread about one month after invasion, which corresponds with the time necessary for symptom development (Fauquet and Fargette, 1990). These examples are therefore an indication that disease spread mightbe facilitated when a high population density of *B. tabaci*, feeds on plants containing a high virus titre and subsequently infects disease-free plants over a large area.

Major pests that affects cassava are the cassava mealybug (*Phenacoccus manihoti*), green spider mite (*Mononychellus tanajo*a) (Akinlosotu,1985) and whitefly (*Bemisia taba*ci) (perrings, 2001). Palaniswami and Pillai (1984) reported negative results when *B. tabaci* were used for transmission experiments from cassava to cassava. Malathi *et al.* (1985) also reported negative transmission of cassava mosaic from cassava to members of *cucurbitaceae* and *solanaceae* by *B. tabaci*, but obtained negligible transmission from cassava to cassava by using high population of *B. tabaci*.

Antony *et al.* (2009) successfully transmitted ICMV from cassava to cassava by cassava biotype *B. tabaci*. They reported that the *B. tabaci* which rear in cassava only transmit CMD from cassava to cassava, the sweet potato biotype *B. tabaci* failed to transmit CMD from cassava to cassava.

Seifi (1981) first reported successful transmission of ACMV by *B. tabaci*. In India, *B. tabaci* was first reported to be the vector of ICMV (Alagianagalingam and Ramakrishnan, 1966; Antony *et al.*, 2006). Mostly Cassava mosaic virus was spread

via cuttings and whitefly transmission (Malathi *et al.*, 1985; Thankappan and Chacko, 1997).

B. tabaci mtCOI haplotypes were examined via Phylogenetic analysis from the African continent has revealed five major cassava-associated haplotypes with the exception of one and two collections from Cameroon and Zimbabwe, respectively (Berry *et al.*, 2004).

Another study showed that both the cassava biotype and sweet potato biotype *B. tabaci* can successfully feed, survive and reproduce on a new host plant species, *Nicotiana debneyi* Domin. These findings have important implications for better understanding the disease epidemics, related to whitefly transmissible *geminiviruses* (Thompson, 2003).

Adjata *et al.* (2012) studied the effect of planting date of cassava on the transmission of cassava mosaic disease by *B. tabaci*. They reported that the whiteflies (*B. tabaci*) are not only vectors of CMD, but also a destructive agent of young cassava seedlings. The propagation of the disease is largely influenced by the date of plantation and this information can be used for the screening of selected cassava clones.

2.6 AGROINOCULATION

2.6.1 *Agrobacterium tumefaciens*

A. tumefaciens, is a gram-negative soil bacterium, a member of the eubacterial family *Rhizobiaceae*, soil phytopathogen, naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. The first evidences indicating this bacterium as the causative agent of the crown gall goes back to more than ninety years (Smith and Townsend, 1907).

2.6.2 *Agrobacterium* mediated gene transfer

A. tumefaciens is mainly used in genetic studies for the introduction of foreign DNA into plants, because it has the ability to transfer Small piece of DNA known as T- DNA (Fig. 6) from the bacteria to the plant cell. A. tumefaciens have Ti-plasmid which has the T DNA, it mainly encode the synthesis of plant regulators auxin and cytokinin. It also encodes the synthesis of many amino acid derivatives, such as opines.

2.6.3 Agroinoculation

Agroinoculation has become an important delivery tool for a variety of viral genomes of interest. Agroinoculation is commonly used for the gene function analyses, mainly for plant virus interactions (Grimsley *et al.*, 1986). As a result of agroinoculation transient gene expression were obtained, which is higher than stable transformation. This transient gene expression is stable for several days (Zottini *et al.*, 2008). Agroinfiltration is a modified form of agroinoculation, which is mainly used to express transgenes in plants for functional analysis and the most common agroinfiltration method using needleless syringe for the introduction of *Agrobacterium* (Wroblewski *et al.*, 2005).

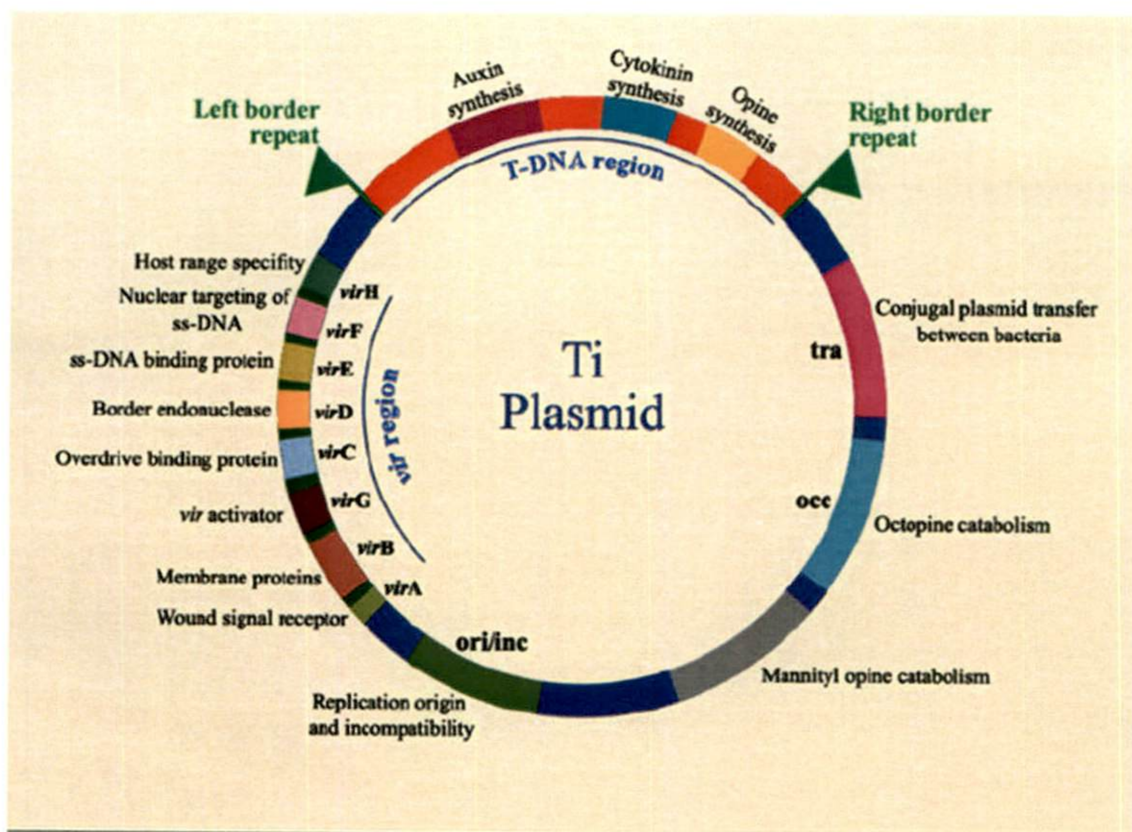


Figure 6: Schematic representation of Ti plasmid of *Agrobacterium tumefaciens*

The first report of agroinoculation in plants was to study *Maize streak virus* and *Cauliflower mosaic virus* (CaMV) (Grimsley *et al.*, 1986 and Grimsley *et al.*, 1987). *Agrobacterium* mediated transient gene expression analysis also used to identify and screen resistance genes by co-expressing a candidate *R* gene with its matching *Avr* gene in plants (Tai *et al.*, 1999 and Bendahmane *et al.*, 2000). Vacuum aided agroinfiltration technique used by Rossi and colleagues (1993) to measure the efficiency of T DNA transfer of *Agrobacterium*.

To study the infectivity, replication and movement of cloned viral genomes agroinoculation has been widely used (Buraguhain *et al.*, 1994; Czosnek *et al.*, 1993; Mandal *et al.*, 1997; Kheyr-Pour *et al.*, 1991).

Only recently, a very efficient agro-inoculation protocol with ACMV-NOg infectious clones was developed for cassava (Vanderschuren *et al.*, 2009). They developed a screening system, which combines the infection assay and CMD resistance-associated molecular markers to identify CMD resistant or susceptible cassava varieties (Huiping *et al.*, 2010).

Agro-inoculation has been successful to introduce geminiviruses into host leaf disks, germinating seeds, and whole plants (Czosnek *et al.*, 1993; Kheyr-Pour *et al.*, 1994).

Biswas and Varma (2001) developed agroinoculation as a useful method for screening germplasm of legumes to *mungbean yellow mosaic geminivirus*. They tested two different methods of agroinoculation, sprouted seed and seedling inoculation, both were found equally effective.

Mittal *et al.* (2008) reported the agroinoculation of cloned SLCMV DNA to the model plants *Nicotiana tabacum* and *Arabidopsis*. This study showed that the accumulation levels of viral DNA in the inoculated plants shows that this virus was poorly adapted to the additional hosts.

Another study showed that a new strains of ICMV causes mosaic disease in *Jatropha curcas*. Agroinfiltration of the two cloned viral DNA components produced systemic infection and typical mosaic symptoms in *J. curcas*, thereby fulfilling Koch's postulates. Thus the availability of infectious clones will provide a valuable tool to screen *J. curcas* cultivars for disease resistance and facilitate the generation of virus-resistant *J. curcas* plants by transgenic technology (Gao *et al.*, 2010).

2.7 BIOLISTIC METHOD

Biolistic inoculation is a direct physical gene transfer method in which nucleic acids are coated on biologically inert microparticles and delivered directly into the nucleus of target cells by high-velocity acceleration

The particle bombardment method was first described by Sanford *et al.* (1987) for the development of transgenic plants and advanced by John Sanford, Ted Klein and colleagues at Cornell University (Sanford *et al.*, 1987; Sanford, 2000). They coined the term 'biolistics'. Heterogeneous tungsten particles are widely consumed as a physical factor but the gold particles in the range of 0.7-1.0 µm mean diameter give more transformation efficiency (Southgate *et al.*, 1995; Taylor and Fauquet, 2002; Sanford *et al.*, 1993; Kikkert, 1993; Sanford *et al.*, 1991). Biolistic method is mainly used for the production of transgenic plants, inoculation of plants with viral pathogens and transient gene expression studies (Sanford, 2000; Southgate *et al.*, 1995; Taylor and Fauquet, 2002).

Biolistic method has many advantages over *Agrobacterium* inoculation. Hypersensitive responses to *Agrobacterium* that leads plant cell death are eliminated (Perl *et al.*, 1996) via biolistics. Also the operation of the biolistic device is easy.

Biolistics also has some disadvantages. It is more costly than agroinoculation and sometimes the transformation efficiency might be lower compared to agroinoculation. DNA damage occurs during biolistics method and intracellular

targets are random. Many researchers reported that biolistic has high frequency for complex integration patterns and multiple copy insertions that could cause variation of transgene expression and gene silencing (Gao *et al.*, 2008; Darbani *et al.*, 2008).

Taylor and Fauquet (2002) develop a new method, Agrolistics, to increase efficiency of original biolistics and yield simpler integration patterns. Use of agrolistics has been shown to increase the number of transgenic plants that have the clean integrate or precisely transgene as well as to reduce the frequency of degraded transgene integrations (Hansen and Chilton, 1996).

Particle bombardment (or biolistic inoculation) is a well-known technique to introduce nucleic acids into plants (Klein *et al.,* 1987; Sanford, 1988). Genome components (DNA-A and DNA-B) of a begomovirus were amplified by RCA. The amplification products were inoculated biolistically into plants and shown to be very effective for inducing infection (Knierim and Maiss, 2007).

Clones of an *African cassava mosaic virus* isolate originating from Nigeria (ACMV-NOg) were shown to be infectious to cassava by biolistic inoculation and this is the first demonstration of infectivity of a cloned geminivirus to cassava and conclusively proves that ACMV is the causative agent of cassava mosaic disease (Briddon *et al.*, 1998).

Cloned components ICMV (isolated from Maharashtra), were tested by biolistic method. These cloned components produced a systemic infection and typical mosaic symptoms in cassava, there by fulfilled the Koch's postulates. The availability of these types of infectious clones will provide a valuable tool to screen new cassava cultivars for disease resistance under defined conditions (Rothenstein, 2005).

Biolistic methods can be used for screening for the tolerance / resistance against geminivirus. This technique is very fast and may speed up the evaluation of

different cassava cultivars to complement breeding programmes (Ayeh and Ramsell, 2008).

Ariyo *et al.* (2003) studied and compared the biolistic inoculation method and graft inoculation approach to deliver DNA. It shows that the infection symptoms were earliest in biolistically inoculated cassava plants. Thus biolistic inoculation of virus-laden DNA extracts is the most efficient virus transmission technique and it is recommended for screening cassava germplasm

2.8 DETECTION OF *CASSAVA MOSAIC VIRUS*

The disease in a field can be observed by visual symptoms and symptoms scoring. But these symptoms are highly variable according to the severity of the disease, the periods of dryness and at the time of mineral deficiency of the cassava seedlings. This limits the disease diagnosis based on symptoms. So it will be confirmed by accurate diagnostic tests.

The Enzyme-Linked Immunosorbant Assay (ELISA) is most widely used for the quantification and detection of virus because this method is very simple and sensitive(Clark and Adams, 1977). The first report of ELISA was used as a novel tool for the detection of geminiviruses in plant by Givord *et al.* (1994). However, this method has certain limitations for the detection of geminiviruses such as its inability to distinguish different cassava mosaic begomovirus in mixed virus infections (Thottappilly *et al.*, 2003).

Ogbe *et al.*(2003) and Nirbhay *et al.* (2010) reported that the polymerase chain reaction (PCR) can also be used for the detection of plant viral genome, which is sensitive than ELISA. Ogbe *et al.* (2006) successfully detect the mixed infections of ACMV and EACMV-Ug. Makeshkumar *et al.* (2005) developed virus specific primers (full length coat protein (CP), replicase (AC1), movement protein (MP) and partial coat protein (CP1)) to detect the ICMV infection in plants.

Materials and methods

3. MATERIALS AND METHODS

The present study was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016.The details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 RAISING HEALTHY CASSAVA PLANTS

Healthy virus free cassava plants were multiplied by *in vitro* propagation. The cassava variety used for this study was H226, which is highly susceptible to cassava mosaic disease. The meristem derived virus free plants maintained at ICAR- CTCRI were sub cultured in MS media (Appendix I) and incubated at 28°C. After rooting, 2 – 3 leaves staged plantlets were hardened in coir pith and used for present study.

3.2 RAISING HEALTHY NICOTIANA BENTHAMIANA PLANTS

The seeds collected from healthy disease free *N. benthamiana* plants and sowed in coir pith. After 3-4 weeks two to three leaves staged seedlings planted in separate pots and placed in an insect proof cage. These plantlets used for the present study.

3.3 WHITEFLY TRANSMISSION

3.3.1 Preparation of insect proof cages

Wooden cages of size 45×45×30 cm were constructed and were covered with muslin cloth on three sides and top with an adhesive fevicol. The front was covered such a way that it could be easily opened with the help of Velcro. Cardboards were used for the base of the cage.

3.3.2 Collection of whiteflies

An aspirator consisting of a glass tube (30 cm length and 0.5 cm diameter) and a rubber tube of 40 cm length was used for collection of whiteflies. The whiteflies were collected from the leaf by turning the leaves upwards and then the whiteflies were aspirate into the glass tube. Whitefly (*Bemisia tabaci*) culture was maintained on healthy cassava plants.

3.3.3 Rearing whiteflies

In each insect proof cage healthy cassava plants grown in polythene bags were kept and pure cultures of *B. tabaci* were released. This insect proof cage was used as the rearing cages for pure virus free whiteflies.

3.3.5 Insect transmission

Insect transmission studies were conducted using the vector, *B. tabaci.* Diseased cassava plants were used as the source for acquisition access feeding by *B. tabaci.* About 300- 500 adults were collected from rearing cages and released into the cages with infected cassava plants and allowed to feed to acquire the virus. Then the viruliferous whiteflies were released on to healthy cassava seedlings covered by small plastic tubes by using an aspirator. The whiteflies were given different period of inoculation access feeding and after that the whiteflies were removed. The inoculated plants were kept in an insect proof cage for symptom production.

3.3.6 Standardization of different parameters for insect transmission

Different parameters affecting the efficiency of insect transmission ie., number of whiteflies and effect of inoculation access period (IAP) on symptoms development were studied.

Experimental design

For the optimization of number of whiteflies required for the effective transmission of cassava mosaic disease, the cassava plants were inoculated with different number of viruliferous whiteflies ie., 10, 20, 30, 40 and 50 .

For standardizing the effect of incubation access period, 10 cassava plants with 3 replicates were incubated with three different incubation access period i.e., 24, 48 and 72.

The un-inoculated plants were served as control. All the plants were kept in an insect proof glass house for observation.

3.4 AGROINOCULATION

3.4.1 *Agrobacterium* strains

Three different *Agrobacterium* strains were used for the present study. LBA4404 and C58 strains were obtained from CTCRI and GV3103 from RGCB, Thiruvananthapuram.

3.4.2 Establishment of *Sri Lankan Cassava Mosaic Vi*rus (SLCMV) infectious clones in *Agrobacterium* strains

For the establishment of SLCMV infectious clones in different *Agrobacterium* strains, mainly, two methods were used, Triparental mating and transformation of competent *Agrobacterium* strains.

3.4.2.1 *Triparental Mating*

Prior to 2 days of triparental mating, the single colony of *Agrobacterium* strain was inoculated into 50 ml LB with Rifampicin (20μg/ml). Then it is incubated at 28°C for 48 hours with continuous shaking until sufficient growth appears. One day before triparental mating, single colonies of donor strains (*Escherichia coli* with

infectious clone SLCMV DNA A and DNA B) were inoculated into 50ml LB with spectinomycin (100µg/ml). On the same day, helper *E.coli.* having the plasmid pRK 2013 was inoculated on LB with kanamycin (50µg/ml). Both the cultures were incubated at 37°C for 24 hrs. On the day of triparental mating, in a sterile culture tube 1 ml each of donor and helper, 2ml recipient cell culture were added and mixed together. From this 100µl of mixed cell suspension was placed on the sterile nylon membrane (1×1cm2) kept on the LA plate without antibiotics. Plates were incubated at 28°C overnight for conjugation. After 16-18 hours the nylon membrane having grown cells was placed in a beaker having 5 ml sterile distilled water. By vigorous shaking, the cells were removed from nylon membrane. From this 100µl cell suspension was spread on LA plate having Rif and Spec. The plates were incubated for 2 days at 28°C for the appearance of trans conjugants. Well isolated single trans conjugant colony was picked up and transferred to fresh LA plates having Rif and Spec and maintained at 28°C. The colonies were confirmed by colony PCR.

3.4.2.2 ***Agrobacterium Transformation of Competent Cells***

Single colony of *Agrobacterium* strains were inoculated in 2 ml YEB media and incubated for at 28°C on shaker overnight. Next day 50µl of overnight culture was reinoculated into 50ml YEB media and incubated at 28°C until the OD_{600} reached 0.6 – 1. The cultures were chilled for 5 minutes. From that 30 ml culture taken and centrifuged at 7000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was re-suspended with 10 ml of ice cold 0.15M NaCl and incubated on ice for 15 min. The cell suspension was centrifuged at 7000rpm for 5 min at 4°C. The supernatant was discarded and the pellet was re-suspended with 1ml of ice cold 20ml $CaCl_2$. 100µl of this competent cells were transferred in a tube and added 1µg of DNA (SLCMV infectious clones), mixed up gently with pipette tip and then left on ice for 30 min. Later the tube was immersed in liquid nitrogen for 1 min for freezing and the it was thawed in 37°C water bath for 1 min. Then 400µl LB was added to the

tubes and incubated horizontally on shaker at 28°C for at least 1 hour. After incubation, the content was spread onto LA with 1% w/v glucose containing Rif and Spec and incubated at 28°C for 2 days. The transformed colonies were analyzed by colony PCR method.

3.4.3 Agroinoculation solution: preparation

The agroinoculation protocol was performed in three consecutive days

Day 1: Preculture

Single colony of *Agrobacterium* strains with infectious clones (SLCMV DNA A and SLCMV DNA B) were inoculated into 3 ml medium and incubated overnight at 28°C.

YEB Medium	: 3ml
Rif 20mg/ml	: 3μl
Spec 100mg/ml	: 3μl
1M mgSO4	: 6μl

Day 2 : Main culture

2μl of the overnight cultures used to inoculate 100 ml medium and incubate at 28°C for overnight.

YEB Medium	: 100 ml
Rif 20mg/ml	: 100μl
Spec 100mg/ml	: 100μl
1M $MgSO_4$	: 200μl

Day 3:

The cells were harvested by centrifugation at 4000g;for 8 min, at room temperature and the pellet was re-suspended in infiltration solution to get required OD 600.

3.4.4 Standardization of different parameters for agroinoculation

Different parameters affecting the efficiency of agroinoculation such as different methods of agroinoculation, effect of *Agrobacterium* strains, effect of temperature and effect of bacterial concentration required for the symptoms development were studied.

Experimental design

For standardizing different methods of agroinoculation, the cassava plants and *N. benthamiana* plants were inoculated with different methods such as agroinfiltration, agro inoculation through making injury in leaf, syringe inoculation in stem / petiole and agro drenching. In all experiments five *Nicotiana benthamiana* plants and cassava plants with five replicates were used.

For standardizing effect of agrobacterium strains, the cassava plants and *N. benthamiana* plants were inoculated with three different agrobacterium strains such as GV3103, C58 and LBA4404 having infectious clones.

These inoculated plants were incubated at different temperature for studying the effect of temperature on agroinoculation. Two different temperature were used for this study, ie., 28°C and 37°C.

For standardizing the optimum bacterial concentration required for the effective agroinoculation, four different bacterial concentration (OD_{600})ie., 1, 2, 3 and 4 were used.

The un-inoculated plants were served as control. All the plants were kept in an insect proof glass house for symptoms development.

3.5 BIOLISTIC DELIVERY OF ROLLING CIRCLE AMPLIFICATION (RCA) PRODUCT

3.5.1 Rolling circle amplification of SLCMV genome

The components of reaction mixture

Phi 29 DNA polymerase buffer(10X)	:2µl
Exo resistant random hexamer primers (500µM)	:2µl
dNTPS (100mM)	:2µl
DNA sample	:2µl

This mixture was incubated at 94°C for 3 min. After cooling, 4 µl of pyro phosphatase (0.1U/µl) and 0.7µl phi 29 DNA polymerase (10U/µl) were added and incubated for 18-20 hours at 30°C followed by heat inactivation at 65°C for 10 min. RCA product were checked in 0.8% agarose gel .

3.6 MOLECULAR ANALYSIS

Genomic DNA from the inoculated plant and plasmid DNA from different *Agrobacterium* strains were isolated and used for PCR analysis.

3.6.1 DNA Isolation

CTAB method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was used for genomic DNA isolation.β-mercaptoethanol was added fresh to the CTAB extraction buffer(Appendix III) to give a final concentration of 0.2 percent (v/v). The solution was heated to 60°C in water bath (ROTEK, India). The samples (100 mg) were chilled and pulverized to a fine powder in liquid nitrogen

using a sterile mortar and pestle and transferred in to a sterile 2 ml centrifuge tubes containing 1 ml of freshly prepared warm extraction buffer. The content was homogenized by gentle inversion. The samples were incubated at 60°C in water bath for 30 min with intermittent shaking. Then it was centrifuged at 10,000 rpm for 10 min at RT. The supernatant was transferred to another sterile eppendorf tubes with a sterile pipette tip. To this 10 µl RNase was added and incubated at 37°C for 1 h. The homogenate was then extracted with an equal volume of 24: 1 (v/v)chloroform/ Isoamyl alcohol and mixed well by inversion for 5-10 min. The homogenate was centrifuged (Hermle, Table top refrigerated centrifuge) at15000 rpm for 10 min at RT. To the aqueous phase, 0.8 volume of chilled isopropanol was added and mixed by inversion. The mixture was then incubated at -20°C for at least 1 h or overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 15000rpm for 10 min at 4°C. The supernatant was decanted and the pellet was washed in0.5 ml ethanol (70 percent) twice, each time centrifuging at 12000 x *g* for 5 min at RT and discarding the supernatant. The pellet was air dried for 30-40 min and dissolved in 50 µl of sterile distilled water. The extracted DNA samples were then stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.6.2 Plasmid isolation

Single colonies of bacteria (*E.coli* with SLCMV DNA A and SLCMV DNA B infectious clones) were inoculated in 3 ml of LB with spec (100mg/ml) and incubated at 37°C, 200 rpm. Then overnight grown culture was centrifuged at 10000 rpm for 10 min at room temperature. To pellet added 100µl P1 buffer (appendix) and vortex the mixture. Next added 10µl RNase (10mg/ml) and incubated for 5 min at room temperature. After incubation 200µl of P2 buffer added to the mixture and incubated on ice for 5 min. then added 150µl P3 buffer and again incubated on ice for 5 min. after incubation the mixture was centrifuged at 10000-15000 rpm for 30 min. the collected supernatant again centrifuged at 10000 rpm for 20 min. then 1 ml of ice cold isopropanaol added to the supernatant and incubated at least 1 hour in -20 C.

then centrifuge at 10000-15000 rpm for 5 min. 500µl Of 70 % ethanol added to the pellet and again centrifuged at10000 rpm for 15 min. The pellet was air dried for 30-40 min and dissolved in 50 µl of sterile distilled water. The extracted DNA samples were then stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.6.3 PCR analysis with CP, MP and multi primers

For doing PCR, total DNA was isolated by CTAB method and PCR amplification (Makeshkumar, *et al*, 2005) was done to detect the presence of SLCMV infection in host plants using different primers viz coat protein gene, multiplex PCR primers

Primers Sequence

CP(H) 5'- AAG CTT TTA ATT GCT GAC CGA -3'

CP(B) 5'-GGA TTC ATG TCG AAG CGA CCA-3'

MP (F) 5'- ATG GAG AATAAT AGT AGC AA -3'

MP (R) 5'- TTA TAC ATT TTT GGA TAC AT -3'

SLCMV-A-F 5' –TGT A AT TCT CAA AAG TTA CAG TCN-3'

ICMV-A-F 5'-GCT GAT TCT GGC ATT TGT AN-3'

I/SLCMV-A-R 5'-ATA TGG ACC ACA TCG TGT CN-3'

PCR analysis with CP specific primer

The components of the mixture were optimized as listed below:

Water	: 18.75μl
10X Taq buffer A	: 2.5 μl
dNTP (10 mM each)	: 0.5 μl
Forward primer (CP (H) (10 pmol μl^{-1}))	: 0.5 μl
Reverse primer (CP (B) (10 pmol μl^{-1}))	: 0.5μl
Template DNA	: 2 μl
Taq DNA polymerase (0.05 U μl^{-1})	:0.25 μl
Total volume	: 25 μl

PCR programme was set with initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72° C for 3 min. Final extension was done at 72 °C for 5min.Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1percent). The gel was viewed under gel documentation system.

PCR analysis with MP specific primer

The components of the mixture were optimized as listed below:

Water	: 13.2 µl
10X Taq buffer A	: 2.5 µl
dNTP (10 mM each)	: 1 µl
Forward primer MP (F)(10 pmolµl^{-1})	: 0.5 µl
Reverse primer MP (R)(10 pmolµl^{-1})	: 0.5µl
Template DNA	: 2 µl
Taq DNA polymerase (0.05 U µl^{-1})	: 0.3µl
Total volume	: 20µl

PCR programme was set with initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s and extension at 72° C for 1 min. Final extension was done at 72 °C for 7min.Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1percent). The gel was viewed under gel documentation system.

PCR analysis with multiplex primer

The components of the mixture were optimized as listed below:

Water	: 12.7 µl
10X Taq buffer A	: 2.5 µl
dNTP (10 mM each)	: 1 µl
Forward (ICMV) primer (10 pmolμl^{-1})	: 0.5 µl
Forward (SLCMV) primer (10 pmolμl^{-1})	:0.5 µl
Reverse (I/SLCMV)primer(10 pmolμl^{-1})	: 0.5µl
Template DNA	: 2 µl
Taq DNA polymerase (0.05 U μl^{-1})	:0.3 µl

Total volume : 20µl

PCR programme was set with initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72° C for 3 min. Final extension was done at 72 °C for 5min.Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with PCR marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1percent). The gel was viewed under gel documentation system.

3.6.4 Gel electrophoresis

PCR product was checked by using horizontal gel electrophoresis unit. 10 µl PCR product was mixed with 2µl loading dye and loaded on agarose gel (1 percent) made of 0.5 X TAE buffer (Appendix III). The gel was run at 5 Vcm^{-1} until the dyes migrated $3/4^{th}$ of the distance through the gel. The gel was visualized and documented under the gel documentation system (Alpha Innotech) using 'Alpha Imager Software'.

3.7 STATISTICAL ANALYSIS

The data were subjected to analysis using the SAS system software version 9.

Results

4. RESULTS

The results of the study entitled " Standardization of virus inoculation method for cassava mosaic disease." conducted at the ICAR-Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2015- 2016 are presented in this chapter.

4.1 MULTIPLICATION OF *IN VITRO* CASSAVA PLANTS

In vitro derived virus free cassava plant was mass multiplied in MS medium. After one week of micro-propagation, the new plantlets developed from the node. Both shooting and rooting happened in the same medium. After 2 weeks, the plantlets become 2-3 leaves stage. This staged plants used for the present study.

4.1.1 Hardening of *in vitro* derived plants

After two weeks, the 2-3 leaves staged *in vitro* plants were removed from the medium and washed with bavistin solution and tap water respectively. Then, the plantlets were planted in a paper cup with coir pith and soil mix for hardening (Fig. 7). These plants were nourished with Hogland's solution once in week. After 3 weeks, the hardened plants removed from the paper cup and transferred to a small pot and placed in a glass house.

4.2 ESTABLISHMENT OF INFECTIOUS CLONE IN AGROBACTERIUM STRAINS

The SLCMV infectious clones obtained from Madurai Kamaraj University (MKU) were transferred from DH5α (*E.coli*) to different *Agrobacterium* strains by triparental method. pRK2013 strain used as a helper strain. Ten different infectious clones available in transgenic lab, ICAR-CTCRI were used for triparental mating and *Agrobacterium* transformation of competent cells. From these, only two infectious

Fig. 7 Hardening of in vitro plants

clones viz., SLCMV (TVM 1) DNA A PD (pkkc24) and SLCMV (TVM 2) DNA B PD (pkkc25), were successfully transformed into three different *Agrobacterium* strains, C58, GV3103 and LBA4404 by these two methods.

The transformed colonies obtained from triparental mating method and transformations of competent cells were plated in a selection medium with Rif (20mg/ml) and Spec(100mg/ml). After 48h incubation, single colonies were obtained (Fig. 8) and these colonies were further confirmed for the presence of infectious clones through PCR with CP and MP primers specific to SLCMV DNA A and SLCMV DNA B components respectively (Fig 9 & 10).

The confirmed colonies were maintained as glycerol stock at -80°C and the cultures were sub cultured once in a month.

4.3 AGROINOCULATION IN *N. BENTHAMIANA*

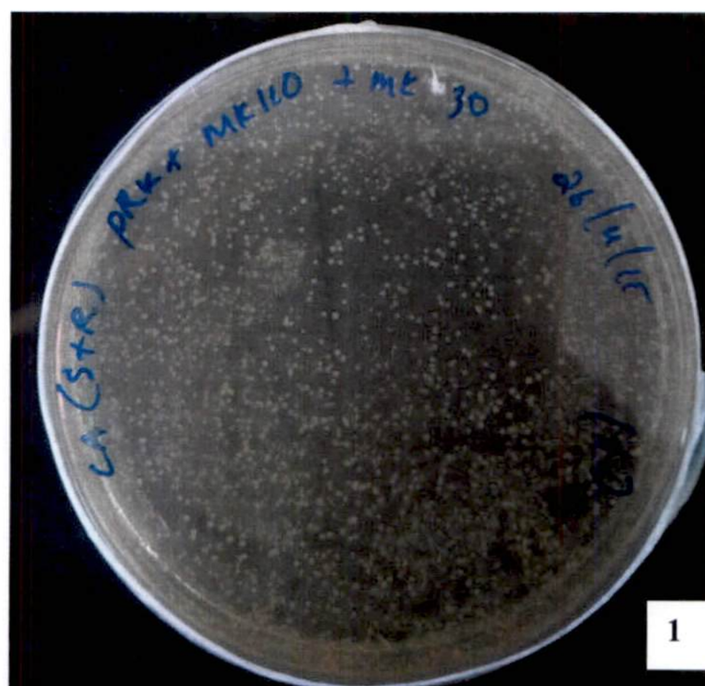
Experiments were carried out to evaluate the different parameters such as *Agrobacterium* strains, *Agrobacterium* growth phase, *Agrobacterium* concentration and temperature on successful agroinoculation in the propagative host.

4.3.1 Agroinoculation in *N. benthamiana*

4.3.1.1 *Effect of Agrobacterium strains on agroinoculation*

Experiments were carried out to evaluate the effect of different *Agrobacterium* strains on agroinoculation. For this three strains were used, LBA4404, GV3103, and C58. Different strains of *Agrobacterium* with infectious clone were agroinoculated in *N. benthamiana*

N. benthamiana plants having 2-3 leaves of each were infiltrated with different *Agrobacterium* solution having DNA A& B. All these plants were kept in an insect proof cage. The responses of the plants were weekly evaluated.



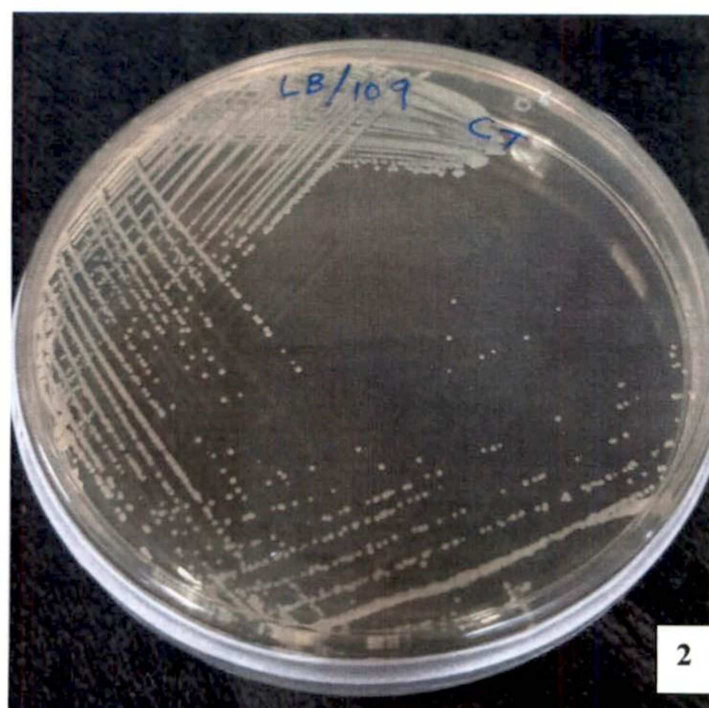


Fig. 8 Establishment of infectious clones in *Agrobacterium* strain, 1: shows the single colonies obtained in the selection media, 2: PCR confirmed colonies of *Agrobacterium* with infectious clones

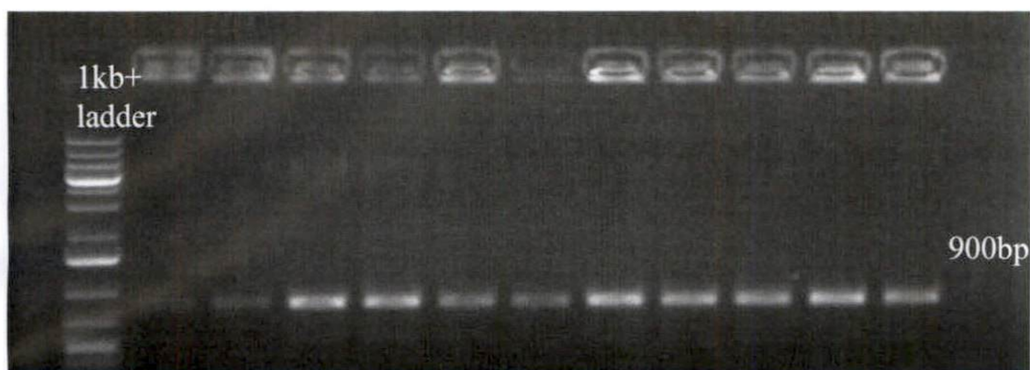


Fig.9. PCR based detection of cassava mosaic virus DNA-B in transformed colonies through colony PCR using movement protein gene specific primer (amplicon size: 900bp)

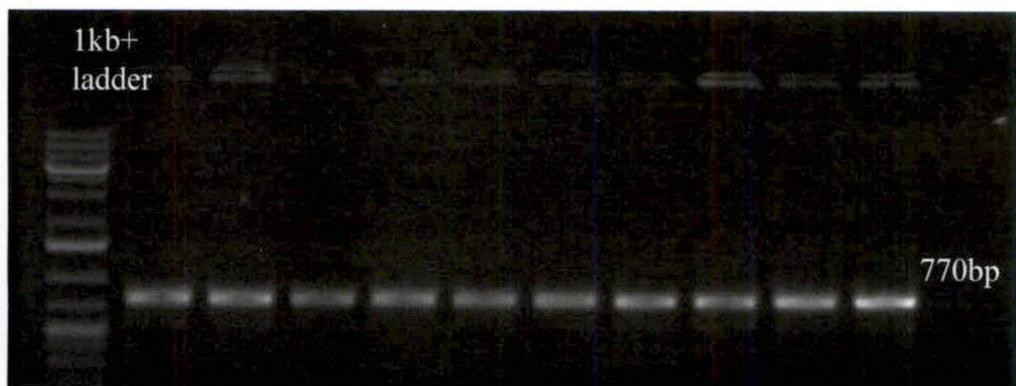


Fig.10. PCR based detection of cassava mosaic virus DNA-A in transformed colonies through colony PCR using coat protein gene specific primer (amplicon size: 770bp)

Comparing different *Agrobacterium* strains, the strain C58 gave the more viral infection symptoms with an average symptom score of 4.8 and the symptoms appeared in 6-7 days after agroinfiltration. LBA4404 strain gave an average symptoms core of 3.2 and these were appeared within 10-12 days. The *Agrobacterium* strain GV3103 showed the minimum symptoms and it took more than 2 weeks for symptoms development (Table 2& Fig. 11)

4.3.1.2 *Effect of bacterial growth phase on agroinoculation*

In order to determine the optimal *Agrobacterium* growth phase , different staged *Agrobacterium* inoculum (OD_{600} values of 0.5-0.8 and 1-1.5) were syringe infiltrated into 4-5 leaves staged *N. benthamiana* plants, which were then grown at 28°C. The resulting CMD symptoms from the different growth phase of agroinocula were shown in table 3.

Comparing these two growth phases, all the three *Agrobacterium* strains at their 0.5-0.8 (OD_{600}) gave the viral infection symptoms within a short time period. The highest transmission efficiency was 92% and 68% with initial OD_{600} of 0.5-0.8 and 1-1.5, respectively. C58 at their OD_{600} 0.5-0.8 gave a symptoms score of 5 in 6 days after agroinfiltration (Fig. 12).

Table 2: Effect of *Agrobacterium* strains on agroinoculation in *N. benthamiana*

Sl. No.	*Agrobacterium* strains	Average mean of symptoms score*	Average mean of % of transmission	Average mean of time taken to express the symptoms (days)
1	C58	4.80	88	6.60
2	LBA4404	3.2	64	10.80
3	GV3103	1.80	32	15.40

* Symptoms score: Hahn *et al.* (1980)

1: unaffected

2: mild chlorosis

3: Pronounced mosaic pattern on most leaves

4: Severe mosaic distortion of two thirds of most leaves, reduction of leaf size

5: Very severe mosaic symptoms on all leaves

The GLM Procedure
Least Squares Means

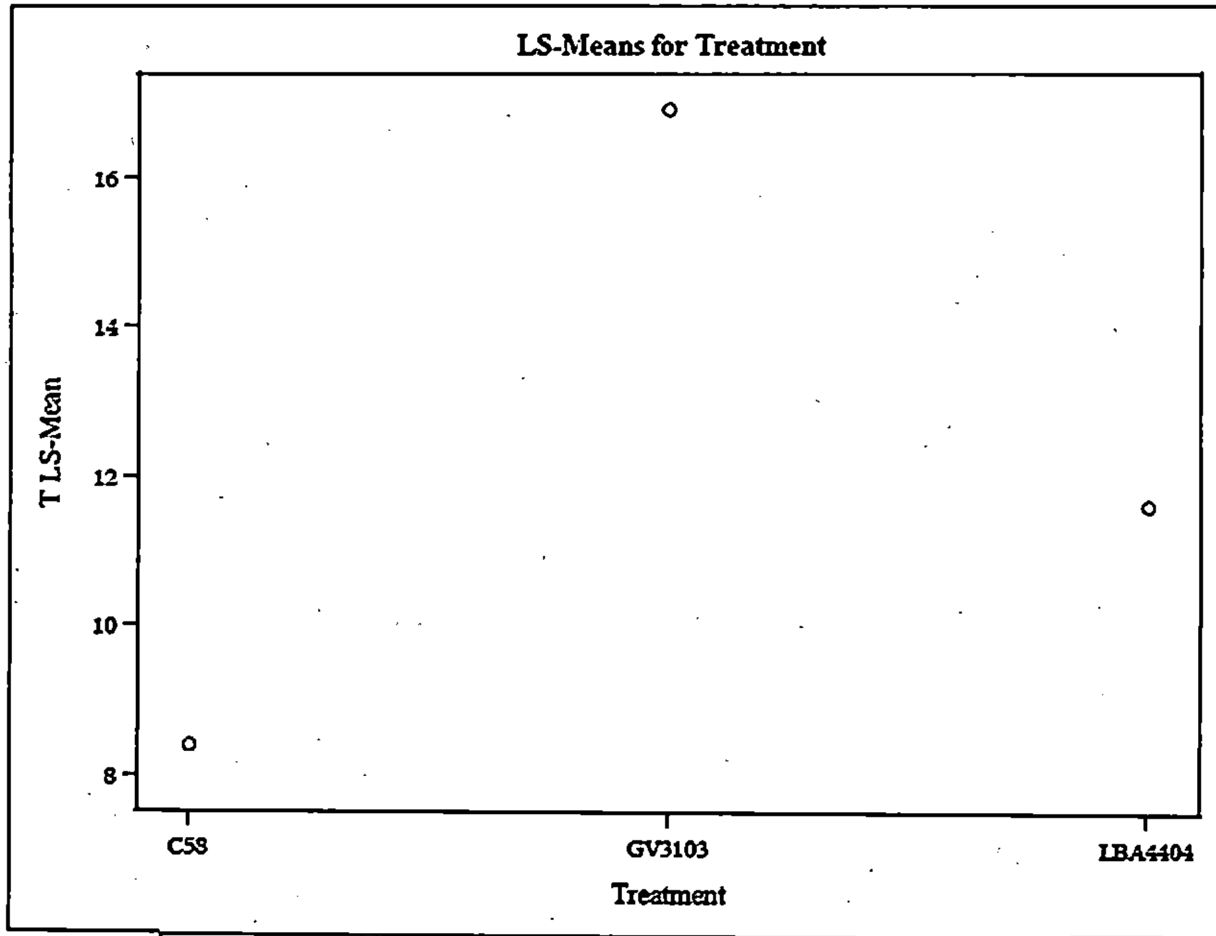


Fig. 11 Effect of *Agrobacterium* strains on agroinoculation

Table 3: Effect of bacterial growth phase on agroinoculation in *N. benthamiana*

Sl. No	*Agrobacterium* strains	OD_{600}nm	Least square means of transmission efficiency	Least square means of symptoms score	Least square means of time taken for expressing symptoms
1	C58	0.5-0.8	92	5	6.4
		1-1.5	68	4	10.4
2	LBA4404	0.5-0.8	64	3	10
		1-1.5	56	2.3	13
3	GV3103	0.5-0.8	36	2	15
		1-1.5	28	1.4	18.8

The GLM Procedure
Least Squares Means

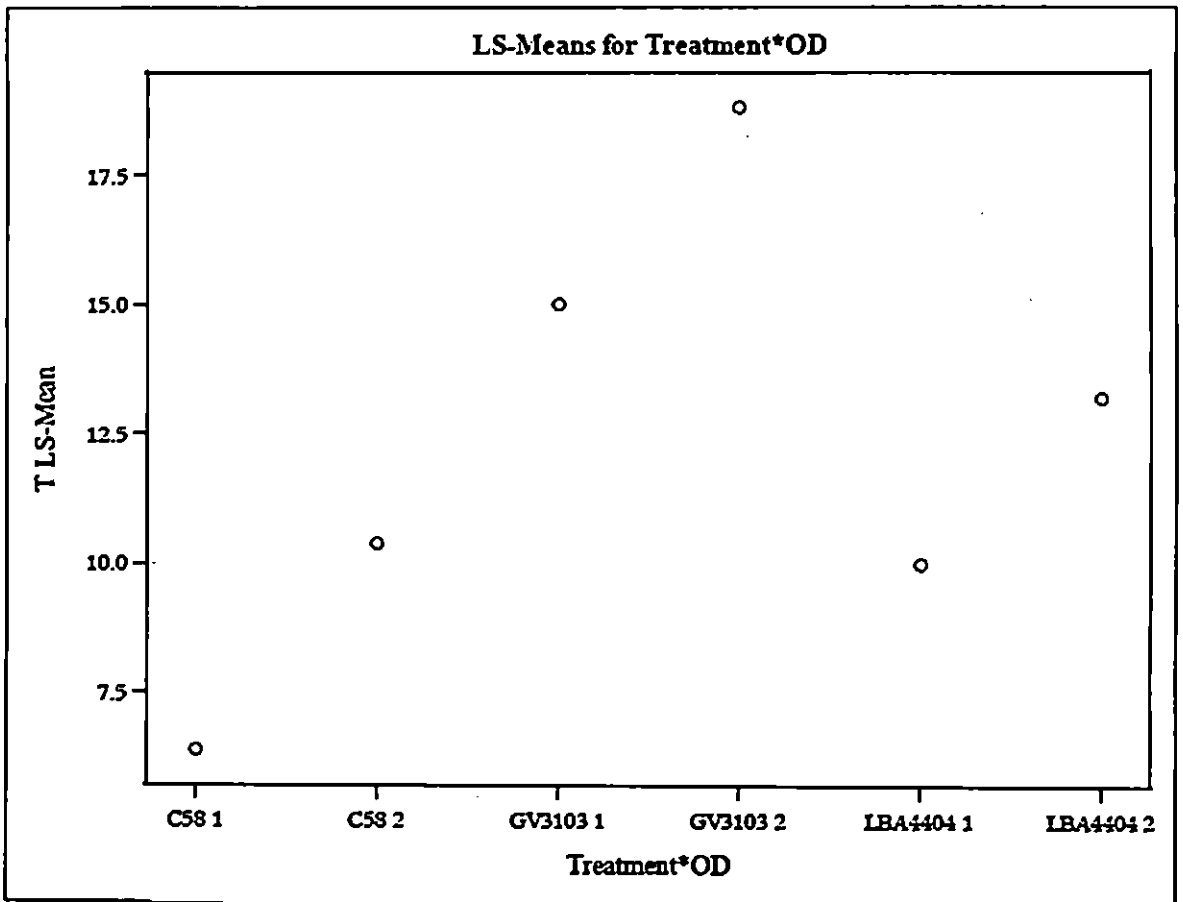


Fig. 12 Effect of *Agrobacterium* growth phase on virus inoculation, 1: OD_{600} 0.5-0.8 and 2: OD_{600} 1-1.5

4.3.1.3 *Effect of bacterial cell concentration on agroinoculation*

In this experiment the effect of bacterial cell concentration at their optimum growth phase were evaluated. For that the bacterial culture when it reached the growth phase OD_{600} 0.5-0.8 were taken and pelletized the cultures. The resulted bacterial cells were re-suspended in infiltration solution and adjusted their cell concentration by checking OD $_{600}$nm at 1, 2, 3, and 4.

The time taken to express the viral infection was decreased when the bacterial cell concentration increased. The bacterial culture at their optimum growth phase gave the maximum infectivity and it is increased with increase in concentration.

The *Agrobacterium* strain C58 gave symptoms score of 5 at their bacterial cell concentration OD_{600} 4 and the symptoms expressed within 6 days. The same strain took 11 days to express the symptoms at their minimal cell concentration (OD_{600} 1). The C58 agrobacterium strains showed 100% transmission efficiency at this cell concentration (Table 4) &(Fig. 13 & 14).

4.3.1.4 *Effect of temperature on agroinoculation*

Agroinoculation was highly depending on temperature. The plants maintained at 28°C were survived and above 37°C, the plants couldn't survive after agroinoculation.

Table 4: Effect of bacterial cell concentration on agroinoculation

Sl. No.	*Agrobacterium* strains	Bacterial cell concentration (OD_{600}nm)	LS mean of transmission (%)	LS mean of symptoms score	LS mean of time taken to express the symptoms (days)
1	C58	1	56	2	11.33
2		2	80	2	8
3		3	83	3	8.33
4		4	100	5	6
5	LBA4404	1	50	2	20
6		2	56	3	13.66
7		3	66	4	11.33
8		4	80	4	10
9	GV3103	1	33	1	25.33
10		2	33	1	20.66
11		3	53	2	19
12		4	63	2	15

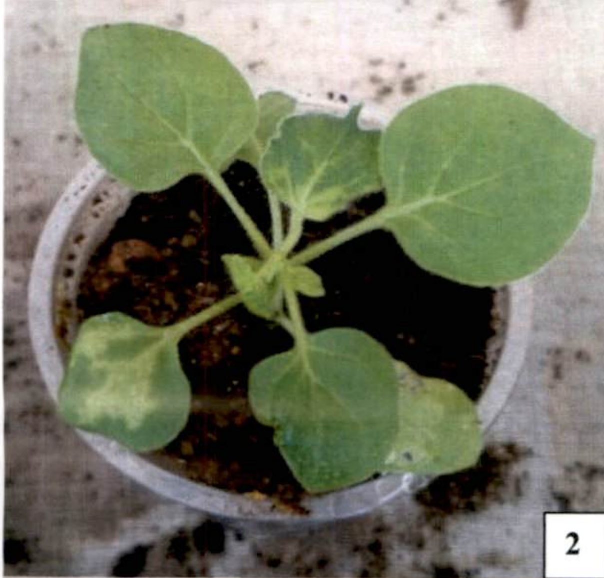


Fig. 13 Symptom expression in the agroinoculated *N. benthamiana* plants, 1: the plants infected with Agrobacterium strain C58, 2: the plants infected with Agrobacterium strain, GV31033: the plants infected with Agrobacterium strain LBA4404

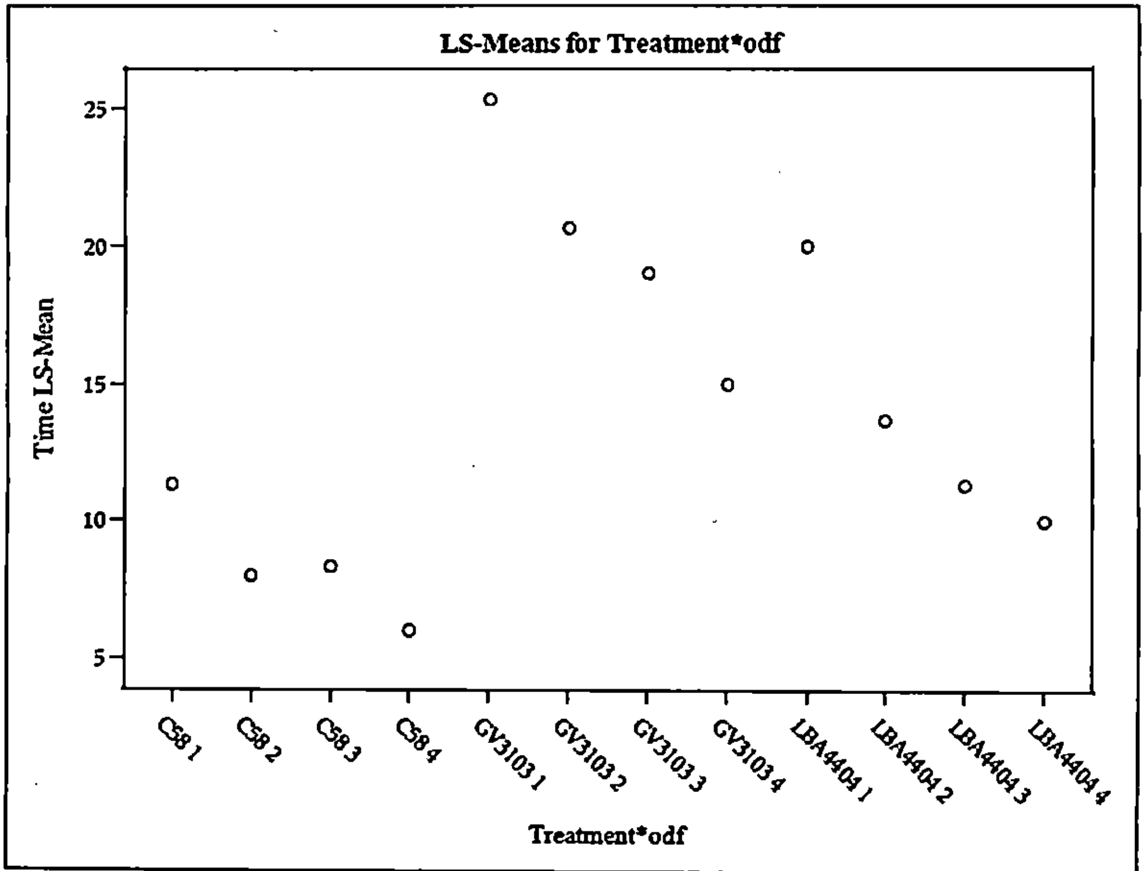


Fig. 14 Effect of bacterial cell concentration on virus inoculation. 1: the agrobacterium culture at initial OD600 0.5-0.8, 2: the agrobacterium culture at initial OD600 1-1.5, T: the time taken to express the CMD symptoms

4.4 AGROINOCULATION IN CASSAVA (H226)

The agroinoculation procedure was standardized in the model plant *N. benthamiana*. The *Agrobacterium* strain C58 at their log phase with a bacterial cell concentration OD_{600}nm 4 gave the best transmission of cassava mosaic virus. This standardized protocol was used for agroinoculation in cassava plantlets.

The *in vitro* derived virus free cassava plants (cv. H226) were used for the present study. Ten cassava plantlets with 5 replicates were used for this study. The dip agroinoculation methods were used in cassava plants.

The cassava plants took more than two months for symptoms development (Fig.15). After agroinoculation the plants were kept in an insect proof cage. The leaves showed mild CMD symptoms were used for cassava mosaic virus confirmation. But it gives a negative result. So the agroinoculation in cassava needs more than one agroinoculation doses.

Total DNA was isolated from the inoculated plants (*N. benthamiana* and cassava) and confirmed the infection through PCR. Only *N. benthamiana* plants showed positive and cassava plants didn't showed amplification. It shows that cassava may require more time for virus inoculum buildup (Fig.16).

4.5 WHITEFLY TRANSMISSION STUDIES

Bemisia tabaci transmitted SLCMV to cassava seedlings up to the extent of 80% with 48 hrs. IAPs and the symptoms appearedin15-20 days after inoculation (Table 5 and Fig. 17 & 18)

Fig. 15 Cassava plants show symptoms after agroinoculation

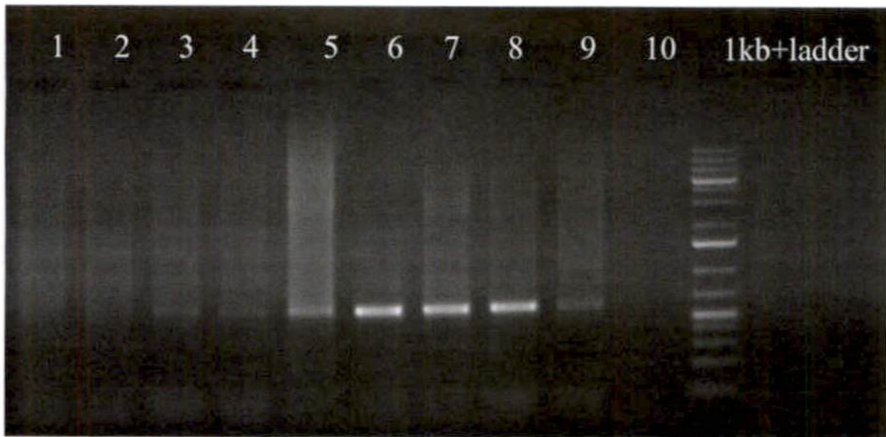


Fig.16 Gel analysis of agroinoculated *N. benthamiana* and cassava plants using multiplex PCR1: control plant 2: agroinoculated cassava, 3 -8: agroinoculated *N. benthamiana* 9: positive control, 10: negative control

Table 5: Effect of number of whiteflies and Inoculation Access Periods (IAP) on whitefly transmission

Sl. No.	No. of whiteflies	IAPs	LS mean of symptoms score	LS mean of time taken to express the symptoms	LS mean of transmission rate (%)
1	5	24	2	28.66	20
2		48	3	25.66	30
3		72	3	25	30
4	10	24	3	23.33	60
5		48	4	18.33	80
6		72	4	19.33	90
7	20	24	3	24	70
8		48	4	20.66	90
9		72	4	20.33	100

Fig. 17 symptoms expression in cassava plants one month after whitefly transmission

The GLM Procedure
Least Squares Means

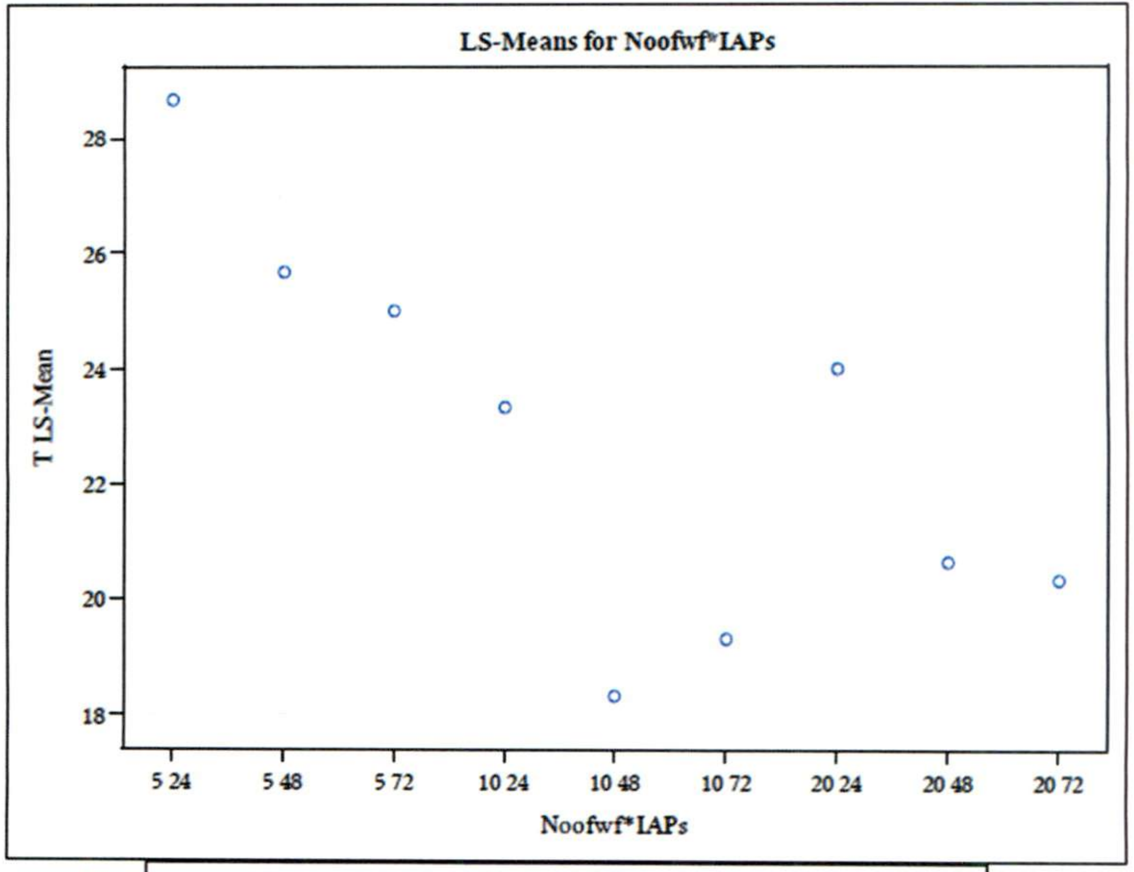


Fig. 18 The effect of no. of whiteflies and IAPs on virus transmission.

4.6 BIOLISTIC DELIVERY

4.6.1 RCA product prepared from SLCMV genome

The SLCMV genome was isolated from the infectious *N. benthamiana* plants and rolling circle amplification was done. The unrestricted RCA product was confirmed by running the product on 1% agarose gel electrophoresis (Fig. 19) and obtained as a smear of concatemers.

The further studies by biolistic delivery couldn't done within the time limit due to technical problems of the instrument (biolistic gene gun which went out of order and could not be made functional till this study completed).

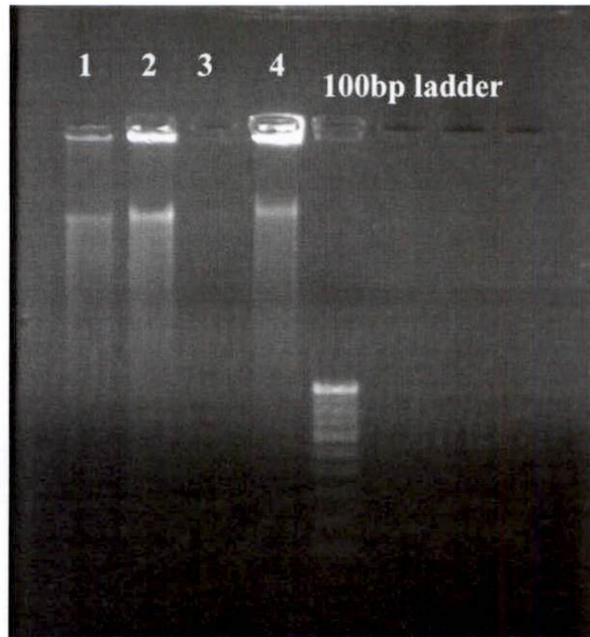


Fig 19 Gel image analysis of unrestricted RCA product run on 1 % gel obtained as a smear of concatemers; 1- 4: SLCMV positive samples

Discussion

5. DISUSSION

Cassava (*Manihot esculenta* Crantz) popularly known as tapioca is an important staple food and an industrial crop, cultivated in 102 countries, from South America(Abraham, 1956). Many reports says that it is introduced into sub-Saharan Africa by the Portuguese mongers (Fauquet and Fargette, 1990), is a major food source of nearly 800 million people in tropical developing countries.

In India it was introduced by the Portuguese from Brazil. Now it is cultivated in about thirteen states with major production in the states of Tamil Nadu and Kerala. The crop integrated very well with the traditions and culture of the people of south Indian states. Adaptability to poor soils, an ability to establish in high and low rainfall areas, and relative resistance to pest and disease are a few factors that greatly support the growth of cassava in these regions. In India cassava is cultivated in 2.28 lakh hectare with a production 81.39 lakh tones (FAOSTAT, 2014).

Like other crops, cassava is vulnerable to many pest and diseases. Many bacteria, fungi and viruses affect the cassava production. Many virus diseases infect cassava crop (Lozano and Booth, 1974). Among the virus diseases affecting cassava crop, cassava mosaic virus is the most important disease in India (Malathi *et al.*, 1985) The natural occurrence of cassava mosaic virus was observed in India by Abraham (1956) and the disease was recognized as a serious threat to cassava cultivation as early as 1942. The disease was described to be widespread in Kerala State, where cassava is mostly cultivated as a subsidiary food crop (Sam Raj, 1966;Menon and Raychaudhuri, 1970). In Karnataka the disease was reported by Muniyappa and Veeresh (1984). In Tamilnadu the disease was reported by Alaglanagalingam and Ramakrishnan in1966.In India CMD causes a yield loss up to 18-88% (Malathi *et al* 1985) and 18 -25% (Anitha *et al* 2011).

CMD produces a variety of foliar symptoms that include mosaic, mottling, misshapen and twisted leaflets, and an overall reduction in size of leaves and plants (Hahn *et al.*, 1980).

The present study was focused on to develop an efficient virus inoculation method for cassava mosaic disease. For that mainly two methods (agroinoculation and whitefly transmission) were compared and standardized. Among them agroinoculation gave the best virus inoculation results. It gave 80-90% virus inoculation efficiency in *N. benthamiana.*

Agroinoculation in *Nicotiana benthamiana* were standardized in this study. Different parameters which affect the agroinoculation were studied. The factors affecting the efficiency of agroinoculation included the *Agrobacterium* strain, *Agrobacterium* growth stage, *Agrobacterium* cell concentration and temperature.

The effect of *Agrobacterium* strain is the most important factor; in the present study only three strains (LBA4404, C58 & GV3103) were used. From this study results, *Agrobacterium* strain C58was the most efficient for agroinoculation in *N. benthamiana* compared to the other strains LBA4404 and GV3103. The *Agrobacterium* strain C58 gave 88%, LBA4404 gave 64% and GV3103 gave 32%. The efficacy of *Agrobacterium* strains differ with respect to the host range and infectious clone. These findings were more or less similar to the earlier reports like, Hosien *et al* (2012) studied the different factors affecting agroinoculation in *Anthurium*. They studied the effect of host factors, *Agrobacterium* factors; media conditions, infiltration time *etc* were studied. They found *agrobacterium* strain GV3101gave 100% efficiency in *Anthurium*. Biswas and Varma (2001) used the agrobacterium strain C58 for screening the germplasm resistance to mungbean yellow mosaic geminivirus.

Another factor which studied was the *Agrobacterium* concentration on agroinoculation. From this study results, the *Agrobacterium* inoculum with an

initialOD600 of 0.5-0.8 and final concentration OD600 of 4 was the most efficient for agroinoculation in *N. benthamiana*. When the initial *Agrobacterium* concentration was higher than 1.0, the efficiency of plant infection was low and the CMD symptom was expressed very late. In contrast, when the final *Agrobacterium* concentration was higher than 2.0, the host plants expressed more CMD symptoms. This is probably because, under these optimum conditions, the infectious cassava mosaic virus is more rapidly available in plant cells, causing increased infection. Also these results showed that the bacterium at their log phase showed more infectivity than at their stationary phase. In their log phase it become more infectious and when the concentration of the log phase bacterial cells increased it gave CMD symptoms within less time.

Temperature is one of the most important factors for good viral spread and effective silencing (Burch-Smith *et al.*, 2004). Several *Solanum* species favor lower temperatures, ranging from 16°C to 20°C (Liu *et al.*, 2002a; Brigneti *et al.*, 2004), but the best results in *N. benthamiana* are achieved at temperatures of 28°C or lower. Therefore, temperature is an important consideration when optimizing agroinoculation in plants species. In the present experiment, it observed that the development of good CMD symptoms on tobacco occurs at 28°C, and it must be grown at 28°C for 2 days after infiltration. The low temperature treatment after injection may actually be one of the contributing reasons for the high efficiency observed (Chung *et al.*, 2004).In fact, plants generally grew more vigorously under cooler conditions. Additionally, Fu *et al.* (2005) showed that conditions of low temperature (15°-18°C) and low humidity (30- 40%) enhanced the silencing of PDS throughout inoculated tomato plants, flowers, and fruits.

Wang *et al* (2013) optimized the virus induced gene silencing in pepper by agroinoculation. They studied the growth stage, *Agrobacterium* concentration and the growth temperature of inoculated plants. They got 88% efficiency at bacterial concentration OD 600 0.8 and 1.

For whitefly transmission studies the pure culture of whitefly was reared on virus free meristem derived *in vitro* cassava plants. Then these virus free whiteflies were allowed to feed on CMD infected cassava plants. These whiteflies were used for transmission studies. The present study showed that, 10 whiteflies with an inoculation period 48 h enough for transmitting cassava mosaic virus from cassava to cassava with 80% transmission efficiency and 72hrs of IAPs gave 90% transmission effiency.

Chant (1958) studied the transmission of cassava mosaic virus by *Bemisia spp*. He reported that the whiteflies require at least 4hrs to acquire the cassava mosaic virus from the young leaves of infected cassava plant and it took another 4 h to become viruliferous. The viruliferous whiteflies took a feeding period of 15 min. He also reported that the longer period of feeding gave more infections and the virus-vector relationships of cassava mosaic virus resembles to that of cotton leaf curl virus. Antony *et al* (2009) reported that cassava biotype whiteflies could transfer cassava mosaic virus with 48 h AAP and 48hrs of IAPs.

Similar finding were reported by Duraisamy *et al* (2012) that whiteflies with 48 h of AAP and 48 h of IAP were transmitted ICMV and SLCMV from cassava to cassava.

After transmission studies the inoculated plants were kept in insect proof glass house. The symptom developments were observed weekly. After symptoms development the presence of virus were detected by PCR with virus specific primers.

Deng *et al* (1994) designed a degenerative primer with an amplification of approx. 500bp and detected whitefly transmitted geminiviruses in plants. They also detected the presence of virus in single viruliferous whitefly. Six virus isolates were detected from leaf curl affected tomato by using these primers.

Summary

6. SUMMARY

The study entitled 'Standardization of virus inoculation method for cassava mosaic disease' was carried out at the Division of Crop Protection, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. The objective of the study was to optimize the virus inoculation procedures for cassava mosaic disease using different methods viz., agro-inoculation, biolistic delivery of rolling circle amplification (RCA) product, whitefly transmission. The important findings of the above studies are summarized in this chapter.

In vitro derived virus free plants were mass multiplied in MS medium. After one week of micro-propagation, the new plantlets developed from the node. After two weeks, the 2-3 leaves staged *in vitro* plants were removed from the medium and washed with bavistin solution and tap water respectively. Then, the plantlets were planted in a paper cup with coir pith and soil mix for hardening.

The SLCMV infectious clones obtained from Madurai Kamaraj University (MKU) were transferred from DH5α (*E.coli*) to different *Agrobacterium* strains by triparental method. pRK2013 strain used as a helper strain. Two infectious clones viz., SLCMV (TVM 1) DNA A PD (pkkc24) and SLCMV (TVM 2) DNA B PD (pkkc25), were successfully transformed into three different *Agrobacterium* strains, C58, GV3103 and LBA4404.

Among the different *Agrobacterium* strains, the strain C58 gave the more viral infection symptoms with an average symptom score of 4.8 and the symptoms appeared in 6-7 days after agroinfiltration. LBA4404 strain gave an average symptoms score of 3.2 and these were appeared within 10-12 days. The *Agrobacterium* strain GV3103 showed the minimum symptoms and it took more than 2 weeks for symptoms development. All the three *Agrobacterium* strains at their 0.5-0.8 (OD_{600}) gave the viral infection symptoms within a short time period. The time

taken to express the viral infection decreased when the *Agrobacterium* cell concentration increased. The bacterial culture at their optimum growth phase gave the maximum infectivity and it increased with increase in concentration. Agroinoculation was highly depending on temperature. The plants maintained at 28°C were survived and above 37°C, the plants couldn't survive after agroinoculation. The dip agroinoculation method was used in cassava plants. The cassava plants took more time for symptoms development.

Bemisia tabaci transmitted SLCMV to cassava seedlings up to the extent of 80%with 48 h IAPs and the symptoms appearedin15-20 days after inoculation. . However in cassava the symptoms could not be observed till the end of this study (~3months after agroinoculation).The SLCMV genome was isolated from the infectious *N. benthamiana* plants and rolling circle amplification was done. Further studies by biolistic delivery couldn't be done within the time limit due to technical problems of the instrument (biolistic gene gun which went out of order and could not be made functional till this study completed).

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Appendices

APPENDIX I

MS MEDIUM

Dissolve 4.4 gm Murashige and Skoog (including vitamins) and 20gm sucrose in 950ml sterile distilled water. The pH was adjusted to 5.8 using 1N NaOH/HCl. Made up the volume to 1 L and 6.8gm Agar was added. Autoclaved at 121°C and 1.06 Kg cm^{-2} pressure for 20 minutes.

APPENDIX II

MEDIUM FOR BACTERIAL CULTURES

1. **Yeast extract broth (YEB)**

Beef extract (Himedia)	3 g/L
Yeast extract (Himedia)	1 g/L
Casein enzyme hydrosylate((Himedia)	5 g/L
Sucrose(DuchefaBiochemie)	5 g/L

pH was adjusted to 7.5 with 1N NaOH/HCl . Sterilized by autoclaving.

2. **Luria Agar (LA)**

Suspended 35.0 grams of LA (HiMedia) in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

APPENDIX III

CTAB DNA Extraction Buffer

CTAB	2%
PVP	2%
Tris- HCl (pH 8.0)	100 mM
EDTA	25 mM
NaCl	2 M
β-mercaptoethanol	0.2 % (v/v) freshly added prior to DNA extraction

APPENDIX IV

INFILTRATION SOLUTION (100ml)

10mM $MgCl_2$	1ml
10mM MES buffer	1ml
150μM Acetosyringone	75μl

MES BUFFER

MES - 9.76g

Sterile dH_2O.- 40ml

Mix well and adjust the pH 5.5 using NaOH pellet. Makeup to 50ml with sterile dH_2O.

APPENDIX V

STOCK PREPARATION

Antibiotic	**Stock solution concentration**	**Solvent**	**Method of sterilization**	**Storage temperature**
Rifampicin	* 20 mg/ml	Methanol	No need to Sterilize	-20°C
Kanamycin	80mg/ml	Sterile distilled water	Filter sterilization	-20°C
Spectinomycin	100mg/ml	Sterile distilled water	Filter sterilization	-20°C
Magnesium Sulphate	1M	Sterile distilled Water	Filter sterilization	-20°C
Acetosyringone*	100mM	DMSO	No need to Sterilize	-20°C

* Light sensitive: container should be covered with foil.

APPENDIX VI

Hoagland's solution

Stock name	Components	g/L	For 1000ml solution
Stock A	KH_2PO_4	136.09	1ml
	KNO_3	101.1	5 ml
	$Ca(NO_3)_2$	164.1	5 ml
	$MgSO_4$	120.39	2 ml
Stock B (micronutrients)	H_3BO_3	2.86	1 ml
	$MnCl_2$	1.81	
	$ZnSO_4.7H_2O$	0.22	
	$CuSO_4.5H_2O$	0.08	
	$H_2MoO_4.H_2O$	0.02	
	D. H_2O	1000 ml	
Stock C (Iron solution)	Iron tartrate	5	1 ml
	D. H_2O	1000ml	

Abstract

STANDARDIZATION OF VIRUS INOCULATION METHOD FOR CASSAVA MOSAIC DISEASE

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Abstract of the thesis

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9. ABSTRACT

The study entitled "Standardization of virus inoculation method for cassava mosaic disease" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. The objective of the study was to optimize the virus inoculation procedures for cassava mosaic disease using different methods viz., agro-inoculation, biolistic delivery of rolling circle amplification (RCA) product and whitefly transmission. *In vitro* derived virus free cassava plants (H226) was used for the study. Two infectious clones namely SLCMV (TVM 1) DNA A PD (pkkc24) and SLCMV (TVM 2) DNA B PD (pkkc25), were successfully transformed into three different *Agrobacterium* strains C58, GV3103 and LBA4404. The virus inoculation method for screening of cassava mosaic disease resistance was standardized and the factors that affect the efficiency of agroinoculation and whitefly transmission were investigated. Consequently, an optimal protocol was obtained by the syringe-infiltration method in the leaves of *N. benthamiana*. The protocol involved inoculation of 4-5 leaf stage *N. benthamiana* plants with the *Agrobacterium* strain C58 inoculum, having an initial OD_{600} 0.5-0.8 and final OD_{600} of 4.0 and incubation at 28°C for 6-7 days showed high cassava mosaic virus transmission efficiency in *N. benthamiana*. Viral symptoms were observed on the leaves of *N. benthamiana* plants 6 days after inoculation, which indicated that this protocol could be used to screen germplasm for their resistance to cassava mosaic disease.