MOLECULAR CHARECTERIZATION OF *SWEET POTATO FEATHERY MOTTLE VIRUS*

by

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(2012-09-114)

THESIS

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DECLARATION

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ASHNA N. K.

DEDICATED TO MY PARENTS
AND BROTHER

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
3'	Three prime
5'	Five prime
A	Adenine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
CI	Cylindrical inclusion
cm	Centimetre
CP	Coat protein
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
DNA	Deoxyribo nucleic acid
DEPC	Diethyl pyrocarbonate
DIBA	Dot Immuno Binding Assay
dNTPs	Deoxy nucleotide tri phosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH
EA	East Africa
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
F	Forward primer

FAOSTAT	Food and Agriculture Organization Statistical Database
g	Gram
G	Guanine
h	Hour
ha	Hectare
HC-Pro	Helper component proteinase
IgG	ImmunoglobulinG
kb	Kilobases
kDa	Kilo Dalton
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
N	North
NASH	Nucleic Acid Spot Hybridisation
NCBI	National Centre for Biological Information
NCM	Nitrocellulose membrane
nm	Nanometer
nts	Nucleotides
°C	Degree Celsius
O	Ordinary Strain
ORF	Open Reading Frames
PCR	Polymerase Chain Reaction
p*M*	Picomolar

R	Reverse primer
Rpm	Revolutions per minute
RT	Reverse transcription
RTC	Root and Tuber crops
RC	Russet Crack
S	South
Sec	Second
SNP	Single nucleotide polymorphism
SPCSV	*Sweet potato chlorotic stunt virus*
SPDFV	*Sweet potato chlorotic fleck virus*
SPFMV	*Sweet potto feathery mottle virus*
SPLCGV	*Sweet potato leaf curl Georgia virus*
SPLCV	*Sweet potato leaf curl virus*
SPLV	*Sweet potato latent virus*
SPMMV	*Sweet potato mild mottle virus*
SPVD	Sweet potato virus disease
ssRNA	Single stranded RNA
TBS	Tris Buffered Saline
T	Thymine
U	Unit
V	Volt
VPg	Viral genome-linked protein

INTRODUCTION

1. INTRODUCTION

Ipomoea batatas (L.) Lam. is a dicotyledonous perennial plant cultivated as an annual crop (family, Convolvulaceae) and is cultivated in more than hundreds of countries as a valuable source of food, animal feed, and industrial raw material. Sweet potato ranks seventh in global food crop production and is the third most important root crop after potato and cassava, principally because of its versatility and adaptability. Nutritional value, resistance to several production stresses and high yield per unit area are the main positive characters of sweet potato (Kays, 2005).

Sweet potato is cultivated on about two lakh hectares of land in India, yielding ca 1 million tons (FAOSTAT, 2015). Approximately 98.5 percent of the world's annual output is produced in developing countries. Out of this, nearly 80 percent is grown in Asia.

The production is effected by several biotic constrains such as insect pest, weeds and crucial viral diseases that leads to the reduction of yield over fifty percentage (Mukasa *et al*., 2006). Devoloping countries that are heavily dependent on food security in agricultural production, export and employment earnings makes plant viruses more economically important. 30 or more viruses have now been observed to effect sweet potato (Clark *et al*., 2012). These viruses are mainly spread through vines which are healthy looking that are collected by farmers from the previous crop for the next section of cropping. Thus, singly infected virus can act as a source of inoculum and the vector transmission leads to mixed infections of different viruses (Rukarwa *et al*., 2010).

Sweet potato virus disease (SPVD) caused by the dual infection and synergistic interaction of *Sweet potato chlorotic stunt virus* (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV) is distributed all over (Mukasa *et al*., 2006). It is the most devastating disease, causing reduction in growth of plant and yield of storage roots (Gibson *et al*., 2004). SPFMV is odserved wherever sweet potatoes are

grown. Eventhugh SPFMV is not a major problem individually, it is devastating in SPVD due to the combained effect of the viruses, causing significant reduction of yield in susceptible varieties (Hanh, 1979). Sweet potatoes infected by multiple virus are now a days a common phenomenon. In India *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato latent virus* (SPLV), *Sweet potato chlorotic fleck virus* (SPDFV) and *Sweet potato leaf curl virus* (SPLCV) are the five main viruses detected to effect sweet potato production (Makeshkumar *et al.,* 2001).

SPFMV is the most dreadful and widespread virus among the sweet potato viruses. It is a typical species of *Potyvirus* genus, one of six genera included in the family *Potyviridae* (Adams *et al.*, 2005). SPFMV discovey was happend in 70 years ago in the United States, and now, it becomes the major sweet potato virus in the world (Tairo *et al.*, 2005).

SPFMV Strains which infects sweet potato in Oceania, world's one among the earliest sweet potato growing areas, and in Southern Africa were isolated and characterized through analysis of the phylogenetic relationship of coat protein (CP) encoding sequences (Rannali *et al.*, 2009). Some important sweet potato production areas, including Asia (Ateka *et al.*, 2007) Oceania (Rannali *et al.*, 2009) and western parts of Africa received very loess attention in the area of molecular characterization

RNA viruses are of great potential for genetic variation, rapid evolution and adaptation. Characterization of the genome provides robust information and they are crucial for designing reliable diagnostic tools and further development of efficient and durable disease control strategies. To ensure the quality and production of sweet potato materials effective management of viral disease is a pre-requisite. To fulfil this requirement the present study was undertaken with an objective to identification of different strains and characterisation of SPFMV.

Findings from the current study can provide a platform for monitoring novel virus species and strains that evolve or are introduced. This will aid in devising management strategies for containing the disease.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS (RTC) AND ITS IMPORTANCE

Tuber crops are underground crops. Plants that produce tubers use these tubers as storage organs, mainly for storing starch. Technically, a tuber is either a modified stem, as in white potato (*Solanum tuberosum*) and cocoyam (*Colocasia esculenta*) also known as a corm, or a modified root such as in sweet potato (*Ipomoea batatas*) and cassava (*Manihot esculenta*). These provides a substantial part of the food supply in the world.

The biological efficiencyof tuber crops is high as producers and are of the carbohydrates than the cereals. They can be used as excellent raw materials for the production of a wide variety of industrial products such as alcohol, starch, sago flour, liquid glucose, vitamin C etc., as well as animal feed (Edison, 2002). Being an important part of the diet, RTCs produce more edible energy per hectare per day than any other crop thus plays an important role in food security, nutrition and climate change adaptation. These crops are prominent income sources through value-addition via processing of food and non food uses and direct sale (Scott *et al.*, 2000).

2.2 SWEET POTATO (*Ipomoea batatas* (L.) Lam)

Sweet potato (*Ipomoea batatas* (L.) Lam) is herbaceous tuber-producing perennial vining dicotyledonous plant in the Morning glory (Convolvulaceae) family that originated in America. It presently cultivated as an important subsistence and food security crop in both subtropical and tropical regions (between 40°N and 32°S latitude). Among 500 species of genus Ipomoea, *Ipomoea batatas* is the only plant that produces roots of edible tubers, thus, it is grown as economically import subsistence food crop (Woolfe, 1992). Its sweet-tasting large starchy tuberous roots are very important as edible crop in Southeast Asia, Oceania and Latin America.

After potato and cassava it becomes the third most valuable root crop grown globally. This food crop have a significantly unrealised potential. The capability of sweet potato to produce high yields of dry matter per unit area of land and labour is achievable under variable farming systems and agro-climates.

Linnaeus in 1753 first described *I. batatas* as *Convolvulus batatus*. Later Lamarck in 1791 classified this species within the genus ipomea on the basis of the surface of the pollan grains and the shape of the stigma; hence named as *Ipomoea batatas* (L.) Lam. There are 13 wild species in section batatus related to sweet potato. The cultivars are approximately 3000 worldwide. Some *Ipomoea batatas* cultivares are ornamental plants; still in horticultural context the name tuberous morning glory may be used. The number of chromosomes in the sweet potato plant is 2n=6x=90; hence, it is hexaploid plant with a basic chromosome number x=15.

It is valued by Subsistence farmers because the crop can produce using few input, have the capability to withstand a stresses such as drought. The crop is often left in the field to harvest accordingly in the absence of frost and it can also be sold for cash (Karyeija *et al.*, 1998). The application of sweet potato in crop rotations is attributed to short growth period and functions as a reserve crop in famine. It grows well and produce high yield in marginal soils, in some areas crop can produce up to three harvests per year (Karyeija *et al.*, 1998). Moreover, the hardiness and flexible adaptability of sweet potato allows it to grow in a wide range of agro-ecological zones (Jana, 1982). The improvement capacity of the crop is high due to different level of diversity (Zhang *et al.*, 2004).

In 2014 total global commercial production of sweet potato was 106 million metric tons, harvested from 8 million hectares (FAOSTAT, 2017). Sweet potato is cultivated on about two lakh hectares of land in India, yielding ca 1 million tons (FOSTAT, 2015). The top ten sweet potato producing states in India and their production status during 2014-2015 is given in table 1.

Table 1: Production of sweet potatoes in top 10 states of India during 2014-2015

States/union territories	Production (000 tonnes)
Odisha	391.40
Uttar Pradesh	331.42
West Bengal	242.38
Madhya Pradesh	60.00
Chattisgarh	37.80
Karnadaka	35.21
Assam	32.86
Nagaland	23.40
Meghalaya	16.30
Tamil Nadu	13.49
Total	11,184.26

Source: National Horticultural Board (NHB), Ministry of Agriculture and Farmers Welfare, Government of India

2.2.1 Morphology

The sweet potato is an herbaceous and perennial plant. It showed different types of growth habits like erect, semi erect, spreading and very spreading. Its root system consists of both fibrous and lateral roots. Fibrous roots functions as absorbing nutrients, water and gives anchoring. Lateral roots that functions as storage roots,

which are photosynthetic products. As the plant matures, thick pencil roots get liignified. The cylindrically shaped stem length, like that of the internodes, depends on available water content in the soil and the diffrent growth habits of the cultivar. The stem colour varies from green to totally pigmented, with anthocyanins according to the sweet potato cultivar. Leaves are alternate heart shaped or palmately lobed. The general shape of sweet potato leaves is found to be round, reniform, cordate, triangular hastate, lobed and almost divided. The flower is bisexual. The pedicel and peduncle colour changes from green to completely purple pigmented. The fruit is capsule, more or less spherical with a terminal tip and can be pubescent or glabrous. It has enlarged, long tapered starch filled edible storage roots with varying flesh and skin colours ranging from white to pink, red, purple and brown, and white to orange and purple.

2.2.2 Nutritional values

Sweet potato is a good energy rich food having 113 cal per 100g, with high carbohydrate content (starch and mostly soluble sugars that give a sweet taste, which is unusual for other starchy foods) and pack a powerful nutitional punch (Woolfe, 1992). They can fulfill our daily needs for vitamin A (400%) in one medium spud, along with a lot of potassium and fiber. Eventhough with fewer colories, more natural sugars are present in sweet potato than regular potato. Sweet potatoes have rich content of fiber, sources of vitamins, (vitamin B, C, and beta-carotene) and amino acids lysine, threonine etc. Since the young leaves and shoots are edible, the entire plant is used for feeding animal. Taiwanese researchers scientific study published in the *Asia Pacific Journal of Clinical Nutrition* in 2007, found that the likeliness of effected with lung cancer is 43 to 65 percent less in people combared those doesn't intake sweet otato leaves. The study also proved that those who had much good intakes of vitamin A, alpha-carotene or beta-carotene had a significantly lower risk of getting lung cancer.

Healthy life style and nutrition can be obtained through consuming plants which produce starchy stems, roots, tubers, rhizomes, and corms. In developing they play a vital role in the diet of people. The other uses include usage as animal feed, manufacturing starch, alcohol, and fermented foods and beverages

2.3 VIRUS DISEASES IN SWEET POTATO

Sweet potato production is restricted by several biotic constraints. According to CIP (International Potato Center), 2000b the damage caused by weevils and viruses are alarming threats that resulted in more serious problems leading massive losses worldwide. Viral diseases occur wherever sweet potato is cultivated. It is a vegetatively propagated crop, and systemic pathogens like viruses can persist and spread over successive crop cycles. Buildup viruses from generation to generation is attributed to the method of vegetative propagation (Okpul *et al.*, 2011). In India, 10-60 percent viral disease incidence was observed in sweet potato fields. The pathogen was identified as *Sweet potato leaf curl Georgia virus* (SPLCGV) and SPFMV (Prasanth and Hegde, 2008). Studies have demonstrated losses of 80-90% in areas affected by virus resulting into severe yield reduction and plant death. The distribution of SPFMV in India was first given by Jain *et al.* 1993 and Jeeva *et al.* 2004a. Different viruses reported to infect sweet potato are given in table 2.

Table 2: List of viruses reported to infect sweet potato

Virus name	**Abb.**	**Family/genus**	**Transmission**	**Reference**
Sweet potato feathery mottle virus	SPFMV	*Potyviridae* (*Potyvirus*)	Aphid (non-persistant)	Sakai *et al.*, 1997
Sweet potato virus G	SPVG	*Potyviridae* (*Potyvirus*)	Aphid (non-persistant)	Colinet *et al.*, 1994

Sweet potato latent virus	SPLV	*Potyviridae* (*Potyvirus*)	Aphid (non-persistant)	Colinet *et al.*, 1994
Sweet potato leaf curl virus	SPLCV	*Geminiviridae* (*Begomovirus*)	Whitefly (persistant)	Lotrakul, 1999
Sweet potato mild speckling virus	SPMSV	*Potyviridae* (*Potyvirus*)	Aphid (non-persistant)	Alvarez *et al.*, 1997
Sweet potato mild mottle virus	SPMMV	*Potyviridae* (*Ipovirus*)	Whitefly (persistant)	Colinet *el al.*, 1996
Sweet potato chlorotic stunt virus	SPCSV	*Closteroviridae* (*Crinivirus*)	Whitefly (non-persistant)	Aritua and Adipala, 2004
Sweet potato collusive virus	SPCV	*Caulimoviridae* (*Cavemovirus*)	*	Paprotka *et al.*, 2010
Sweet potato virus 2	SPV2	*Potyviridae* (*Potyvirus*)	Aphid (non-persistant)	Lozano *et al.*, 2009
Sweet potato virus C	SPVC	*Potyviridae* (*Potyvirus*)	Aphid (non-persistant)	Luan *et al.*, 2007
Sweet potato symptomless virus 1	SPSMV1	*Geminiviridae* (*Mastrevirus*)	*	Lozano *et al.*, 2009
Sweet potato chlorotic fleck virus	SPCFV	*Betaflexiviridae* (*Carlavirus*)	*	Lozano *et al.*, 2009
Sweet potato leaf curl Georgia virus	SPLCGV	*Gminiviridae*	Whiteflies	Lotrakul *et al.*, 2003
Sweet potato pakakuy virus	SPPV	*Caulimoviridae* (*Badnavirus*)	*	Kreuze *et al.*, 2009
Sweet potato C6 virus	SPC6V	*Betaflexiviridae* (*Carlavirus*)	*	Lobeenstein *et al.*, 2009

2.4 POTYVIRUS

Potyviruses comprise to the family *Potyviridae*, together with seven other genera. Agriculturally, economically and biologically these are an important plant virus group. They are majorly found in cultivated areas throughout the world, but as recent metagenomics studies have revealed their presence in wild also (Roossinck, 2012). The largest genus in the family potyviridae is the potyvirus of positive sense, single stranded RNA (ssRNA) plant virus currently recognised, many of which contribute to significant losses in agricultural, horticultureal, pasture and ornamental crops. There are 1000 known species in this genus. These characteristics of the virions are of flexous, non-enveloped, filamentous particles, 720-850 nm in length and are transmitted by aphids.Virus remains infectious in the stylet of aphids only for a short duration of time. Their easy transmission is by mechanical means (Ng and Falk, 2006).

The genome of potyviruses is a positive-sense ssRNA molecule with its 5' end covalently linked to a virus-encoded protein viral genome-linked protein (VPg) and its 3' end polyadenylated. Potyviruses share similarity in terms of their genetic structure and expression strategy, to the plant bipartite como- and nepoviruses and to animal picornaviruses. The distinguished characteristic of virus is the cytoplasmic cylindrical inclusion (CI) bodies encoded in cytoplasm of virus (Van Regermortel *et al.*, 2000).

Study of genetic structure and virus diversity is essential for the understanding the molecular evolutionary background in accordense to their virulency, dispersion, and emergence of epidemics. In most of the sweet potato fields, potyviruses are endemic. Furthermore, new viral strains may emerge due to the co-infection of sweet potato potyviruses. The divergence of several positive-sense RNA viruses, including

potyviruses is due to genetic recombination which is an important evolutionary force behind (Chare and Holmes, 2006).

2.5 SWEET POTATO VIRUS DISEASE (SPVD)

Sweet potato virus disease (SPVD), the most harmful disease of sweet potato, severely reducing the yield of affected plants is caused by the synergistic interaction of the aphid-transmitted *Sweet potato feathery mottle virus* (SPFMV) and whitefly-transmitted *Sweet potato chlorotic stunt virus* (SPCSV). SPFMV did not significantly affect the yield but co-infection by SPCSV of these cultivars was associated with enhanced yield minimization. SPVD is a result of double infection of two viruses,which is important because of the greater yield loss than for either alone. The yield losses of up to 90% have been reported in plants affected with SPVD (Gutie´rrez *et al.*, 2003).

SPVD spreads very fast from field to field which bears a diverse host of symptoms, but usually manifests itself in plant showing stunted growth and leaves projecting abnormal smallness or narrowness, distortion or crinkling, pale coloration and mosaic patterns (Gibson *et al.*, 1998). Total yield loss of these plants vary among; anywhere from half its original value to almost zero yield (Mukasa *et al., 2006).* Because of the variability in the presence of the SPVD-associated viruses in certain parts of the world, the effects of dual infection in sweet potatoes vary to display more or less damage and yield loss in crops.

2.6 SWEET POTATO FEATHERY MOTTLE VIRUS (SPFMV)

SPFMV (genus *Potyvirus* and family *Potyviridae*) is world wide common one among the sweet potato viruses (Moyer and Salazar, 1989). Its genomes comprises a positive sense linear single stranded ssRNA with 10.8 kb and 3' end contains a poly (A) region (Sakai *et al.*, 1997). The average *Potyvirus* genome size 9.7 kb and SPFMV genome is larger than that (Shukla *et al.*, 1994). Exceptionally large coat

protein (CP) size of 38 kda in comparison with other Potyviruses is other major speciality of SPFMV (Abad *et al.*, 1992). The genome has a single ORF, flanked by an un-transcribed region (UTR) in both 5'-end and 3'-end encoding a large polyprotein like many potyviruses.

2.6.1 Symptomatology and Host range

SPFMV Symptoms on the leaves are normally slight or absent and sometimes more prevalent on older leaves. Many sweet potato varieties develop only transient, mild symptoms. Reduced crop growth vigour in the early development phase of plant is common but not identified until infected material is grown alongside pathogen tested material. The irregular classical patterns of chlorosis known as feathering along veins of leaves and distinct-to-faint chlorotic spots having no margins to margins of purple colour and on young leaves in some cultivar mild mottle also occur (Makeshkumar *et al.*, 2001; Jeeva *et al.*, 2004).

Symptoms depend on the sweetpotato variety, its age, and the conditions under which it is growing; they also depend on the severity of the SPFMV strain, and whether it is alone or present in the plant with other viruses. The symptoms on foliage are influenced by susceptibility of cultivar, degree of stress, stage of growth, and strain virulence. Symptom expressions are due to increased stress, whereas rapid growth may cause in symptom reduction (Clark and Moyer, 1988).

Transmission by a vector that can pierce or damage a plant and create an entry point for the virus but the virus entry into plant cells is limited by the presence of a cell wall thus it is only possible by mechanical transmission. Through the stylet tips of aphids SPFMV transmitted in non-persistent way as they bite the sweet potato plant. SPFMV effect vast species of sweet potato plants. It include many *Ipomoea* spp. (*I. aquatica, I. alba, I. cordatotriloba, I. heredifolia, I. nil, I. lacunose, I. purpurea, I. tricolor*), *Chenopodium quinoa*, three *Nicotiana* spp. (*N. rustica, N. benthamiana, N. tabacum*), and *Datura stramonium*. The virus not infect reproductive

tissue, so from parent plants it is not transmitted via their seeds but mainly infect vegetative tissue.

1. Yellow spots on leaves.

2. Ring spots

3. Purple feathery like patterns

4. Vein clearing

Figure 1: Symptoms of *Sweet potato feathery mottle virus* on plant leaves

2.6.2 Vector transmission

SPFMV is transmitted by a large number of aphid species in non-persistent manner (Pozzer *et al.*, 1995) and also by vegetative propagation. It is transmitted by several genera of aphids including the cotton aphids (*Aphis gossypii*) and the green peach aphid (*Myzuspersicae*) in non-persistent manner (Brunt *et al.*, 1996).

Aphids, from the family of Aphididae, which may transmit about fifty percent of known insect-vectored plant viruses, are economically important in temperate regions mainly because of their role as plant virus vectors. Aphids possess special biological characteristics like morphs which are specialized to adapt different functions, the alternation of host plant and short life cycles due to parthenogenetic reproduction. Effective acquiring and transmitting ov plant viruses is done through their pecculiar host-finding behaviour that involves sap sampling (wosula *et al.*, 2012). The sap-sampling behavior involves brief probes into the epidermal cells that may last for just a few seconds to determine acceptance or rejection of a plant for feeding. Because of their higher titers in epidermal and sub-epidermal plant cells this behaviour mainly helps transmission of non-persistent viruses (Powell *et al.*, 2006)

2.6.3 Function of Genes

SPFMV is the most thoroughly characterized sweet potato virus (Brunt *et al.*, 1990). SPFMV has many similarities with potyviruses like the biological and cytopathic characteristics, including aphid transmission, presence of pinwheel inclusions and of relatively narrow host range.

The genome of is a single open reading frame, followed by a 3' UTR and a poly (A) tail. The structure of 3' UTR is secondary and it function as recognition of viral replicase. A 3' poly (A) sequences is present in all potyviruses, but they are void of the poly (A) tail addition cellular signal sequence. The genes present in the genome combrises P1, HC-Pro (helper component proteinase), P3, 6K1, CI, 6K2, NIa, NIb, and the coat protein cistron, which is found in a variety of other viruses. The genome of all potyviruses including SPFMV possess only one open reading frame, is shown in figure 2 and the encoded functions of different genes are represented in table 3.

During replication, the entire genome is translated as a polyprotein and cleaved. The gene for cistron is located near the 3' terminal end (Mori *et al.*, 1994). The functions of some of these proteins, including P1, P3 and the two 6K proteins, are not known in detail.

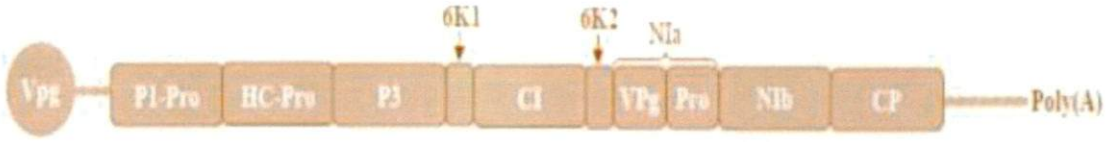


Figure 2: The genome of potyviruses including SPFMV

Table 3: The encoded functions of different proteins in SPFMV

Protein	**Protein function**
P1	Proteinase, ssRNA binding activity
HC-Pro	Proteinase, aids in transmission by aphid, long-distance movement
P3	Viral replication
6K1	Unknown
CI	Cytoplasmic inclusion protein, RNA helicase activity
6K2	Unknown (involved in Viral replication)
Nia	Vpg (viral genome linked protein) and Proteinase
Nib	Viral replicase
Cistron	CP, assembly of virion, control of viral transmission, virus spread

The coat protein (CP) regions are mostly been using for the molecular taxonomy studies of potyviruses. The classification of SPFMV isolates into strain groups is based on their 3' –terminal nucleotide sequences, which also include the CP genes (Kreuze *et al.*, 2000). To understand the change in virulence of viruses and geographical distribution, and how new viral epidemics can emerge, these studies are necessary

SPFMV consists of four strains: common (C), East-African (EA), ordinary (O), and russet cracks (RC) and their phylogenetic analysis of the CP encoding sequences makes them easily identifiable (Kreuze *et al.*, 2000). Tairo *et al.* (2005) reported that Strain C is phylogenetically distant from three of them and a separate virus classification as has been suggested.

There is a classification of SPFMV into three representative strains: Russet Crack (RC), Ordinary (O), and East Africa (Kreuze *et al.*, 2000; Kwak *et al.*, 2007). Strain C was reclassified as a new species, SPVC, by the International Committee on Taxonomy of Viruses in 2010 due to the relatively low homology with the other SPFMV strains (Untiveros *et al.*, 2010). One of the scientific study showed that Korean SPFMV isolates are similar to the RC and O strain isolates, but they are not similar to EA-like isolates (Kwak *et al.*, 2007). EA strain has been detected as a distinct group in East Africa (Mukasa *et al.*, 2003). Strain EA is alsot has found in Vietnam (Ha *et al.*, 2008), Peru (Untiveros *et al.*, 2008), Easter Island (Rännäli *et al.*, 2009) and, very recently, in China (Qin *et al.*, 2013). The other potyviruses that infect sweet potato like SPV2, SPVG and SPLV etc have not been studied exclusively, but recently their complete genome sequences were reported and compared to SPFMV (Ateka *et al.*, 2007; Li *et al.*, 2012; Wang *et al.*, 2013). Internal cork or external cracks on the tubers caused by certain strains of SPFMV result in a total loss of the product quality in market (Moyer, 1988).

2.6.4 Diagnosis and Characterization

The virus is readily detected and identified by several serological techniques including Immunosorbent Electron Microscopy (ISEM), Double Antibody Sandwich Elisa (DAS) and Nitrocellulose Memdrane-Enzyme linked Immunosorbent Assay (NCM-ELISA). Detection of virus in sweet potatoes, however, is more difficult, especially in symptomless tissues or plants (Abad and Moyer, 1992). Some of the major limitations of serological methods like ELISA is like high cost, extensive labour, low sensitivity and higher duration outside the plants vegetative period because of low titre values of the pathogens. Because of frequent serological cross reactions between different species the serological diagnosis of potyviruses is not often pricise (Brunt *et al.*, 1992) and it makes biological indexing a cumbersome process. The detection of plant virus has changed its ways with the large scale improvement in the field of molecular biology, nucleic acid –based methods such as RT and the PCR began to be used (Hsu *et al.*, 2005). Colinet *et al.* (1998) reported that SPFMV can be detected and identified by PCR using virus or genus-specific degenerate primers (Hegde *et al.*, 2012).

Many viruses that infect sweet potato have been now identified and partially characterized at the molecular level, especially in the United States, Australasia, Peru, and East Africa (Souto *et al.*, 2003; Tairo *et al.*, 2005). Strains of SPFMV were isolated and phylogenetically characterized by analysis of the CP encoding sequences in Oceania, one of the world's earliest sweet potato growing areas, and in southern Africa (Rannali *et al.*, 2009). Eventhough, their molecular characterization has got much less attention in some other important sweet potato production areas, like Asia (Ateka *et al.*, 2007) Oceania (Rannali *et al.*, 2009) and western parts of Africa.

2.7 PREVENTION AND CONTROL

The control of the aphid vectors in field is not economically feasible, eventhough their transmission is non-persistent by aphids. Some of the main control measures are sanitation, production and use of virus-free planting material, or use of resistant varieties (Karyeija *et al.*, 2000; Kai *et al.*, 2000; Iwanami, 2004; Gibson *et al.*, 2004). Meristem tip culture is a best method to obtain virus-free plants in many countries (Jeeva *et al.*, 2004 and Zhang *et al.*, 2005). There are two recessive geneswhich confer resistance to SPFMV (Mwanga *et al.*, 2002). SPFMV is perpetuated between cropping cycles in infected cuttings and for the farmers the lack of symptoms in the foliage makes difficulty to select SPFMV-free cuttings. Those wild species of Ipomoea acting as reservoirs of SPFMV should be removed from the field (Clark *et al.*, 1986). The better possibility of transgenic resistant plants be useful in limiting the deleterious effects of SPFMV in future (Okada *et al.*, 2002 and Wambugu, 2003).

One of the best control method used is the genetic method, by which varieties resistant to known viruses are developed. It is done in selected cultivated varieties having an agronomic importance by introducing resistance genes. To facilitate the gene transfer toward the cultivated variety, the sources of resistance are searched for in primary gene pool of the species.

2.8 METHODS OF SPFMV DETECTION

Serological methods like ELISA and nucleic acid based detection like PCR is very efficient accurate in virus detection. In order to improve sweet potato production and to ensure the quality of planting material, effective diagnoses have to done.

2.8.1 Enzyme linked immunosorbent assay (ELISA)

Since 1970, ELISA have been used widely and successfully for the detection of plant viral diseases (Clark and Adams, 1977). ELISA is a solid phase

heterogeneous immunoassay done in microtitre plates made up of polystyrene or polyvinyl chloride. ELISA can be categorized in to two: direct and indirect procedures where ther differ in the way the antigen-antibody complex are detected. The major limitatiof of ELISA is its low sensitivity during periods of low virus tittre. The SPFMV can be diagnosed by ELISA and antisera are commercially available and NCM-ELISA kit has been developed by International potato centre, Peru to detect many of the comman sweet potato viruses. Occurrence of SPFMV in Indian sweet potato cultivars was detected using antisera supplied by International potato centre (Makeshkumar *et al.*, 2001).

2.8.2 Polymerase chain reaction (PCR)

Since 1990 PCR methods for virus identification were published, used (Vunsh *et al.*, 1990) and offered the user theoretically the exquisite levels of specificity and sensitivity utilising gel electrophoresis for resolution of the results. Advancement in the field of molecular biology improvise the plant virus detection with the help ofnucleic methods such as RT and PCR (Hsu *et al.*, 2005). The recognisation of conserved regions of the viral genomes of many virus species or the whole virus genus family is possible through the designed degenerate primers (Posthuma *et al.*, 2002).

The biological databases containing available potyvirus sequences allowed the method development, based upon the PCR for the potyviruses identification (Langeveld *et al.*, 1991). Local conserved regions in potyvirus coat protein were used for the constructing degenerate primers for amplification in a potyvirus group specific combined assay of RT-PCR (Babu *et al.*, 2012). Potyvirus degenerate primers MJ1/MJ2 designed to amplify motifs MVWCIEN to QMKAAA in the core of the CP of potyviruses showed that the region is highly conserved and the respective primers are universal potyvirus group specific (Marie-jeanne *et al.*, 2000; Babu *et al.*, 2012).

Due to the characteristic poly (A) tail at the 3' end of the potyvirus genome, the first-strand cDNA of potyviiruses was synthesised using Oligo-(dT) 12-18 primers or random hexamer with RNA as the template. Hsu et al. 2005 developed RT-PCR method, having the potential to detect members of the genus potyvirus with the help of potyvirus degenerate primers. It is possible to detect many virus species of the same genus or family by combining the RT-PCR technique and degenerate primers, in a single test, but it cannot distinguish the virus species. Now a days, plant viruses are rapidly detectd and identified based on ELISA, RT-PCR with specific primers, or cloning and sequencing methods.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Molecular characterisation of *Sweet potato feathery mottle virus*" is carried out at the Division of Crop protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017. Details pertaining to the experimental material and procedures used in the study are elaborated in this chapter.

3.1 SAMPLE COLLECTION

Sweet potato samples belonging to various accessions were collected randomly from the germplasm collection maintained at ICAR-CTCRI, Thiruvananthapuram. Forty five leaf samples exhibiting viral symptoms (The irregular classical patterns of chlorosis known as feathering along veins of leaves and distinct-to-faint chlorotic spots having no margins to margins of purple colour and on young leaves in some cultivar mild mottle also occur) were collected. The samples were photographed, symptoms were recorded and put in sterile polythene bags and stored at -80°C for subsequent study.

3.2 NUCLIEC ACID EXTRACTION

For carrying out RT-PCR based detection of the Sweet potato feathery mottle viruses infecting sweet potato, RNA isolation is a prerequisite. RT-PCR was performed on RNA isolated from infected samples using genus specific and virus specific primers.

3.2.1 **Isolation of total RNA**

Leaf samples of SPFMV infecting sweet potato plants showing symptoms were taken for RNA isolation. RNA was isolated using three protocols viz., Tri-reagent and Lithium chloride method. RNA pellet was solubilized in 50µl of DEPC treated water and stored at -20°C.

3.2.1.1 **RNA isolation using TRizol reagent**

TRIzol reagent (SIGMA) was used for extraction of total RNA. Samples (80-100 mg) were ground into fine powder in liquid nitrogen using mortar and pestle. The ground sample was immediately homogenized in 1ml of reagent. Mixture was transferred into a micro centrifuge tube and centrifuged at 12000 x g (Hemle, table top refrigerated centrifuge) for 10 min at 4°C and supernatant was collected. Followed by 5 min incubation (Lab ROTEK, India) at room temperature, 0.2 ml of chloroform was added and the tubes were shaken vigorously for 15s. The tubes were incubated for 15 min at room temperature. The samples were centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase of the sample was transferred into a fresh tube. 0.5 ml of chilled 100 percent isopropanol was added to the aqueous phase and mildly inverted to mix and incubated at room temperature for 10 min. The sample was then centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed leaving only a pellet. The pellet was washed with 1 ml of 75 percent ethanol. Discarded the supernatant and the RNA pellet was air dried for 10-15 min. The RNA pellet was then resuspended in 50 µl RNase free sterile water followed by incubation in a water bath at 55-60 °C for 10 min. The RNA was stored at -20°c (Vest frost Low Temperature Cabinet, India).

3.2.1.2 **RNA isolation using Lithium chloride method**

Around 200 mg of leaf samples was ground into fine powder in liquid nitrogen and transferred into a fresh tube and 1 ml of CTAB RNA extraction buffer (pre-warmed at 65°C) (Appendix 1) was added , vortexed and incubated at 65° C (Lab ROTEK, India) for 10 min. The tube was centrifuged at 15,000 rpm (Hemle, table top refrigerated centrifuge) for 15 min at room temperature and supernatant was transferred to a fresh 2 ml tube. Equal volume of chloroform: isoamyl alcohol (24:1) (Appendix 2) was added to the supernatant centrifuged at 20,000 x g for 10 min at 4°C. After transferring the supernatant into fresh tube, equal volume of chloroform:

isoamyl alcohol was added to the supernatant. After centrifugation at 20,000 x g for 10 min at 4°C, the supernatant was transferred to a fresh tube. Then, 0.25 volume of ice cold 10 M lithium chloride was added, mixed well and incubated overnight at 20°C. Following centrifugation at 30,000 x g for 30 min at 4°C, the pellet was washed with 75 percent ethanol by centrifugation at 10,000 x g at 4°C. The washing was repeated two times. The RNA pellet obtained was air dried at 37°C for 30 min for 1 hr while tapping intermittently; added 30-50 µl of DEPC water (Appendix 4) (the RNA and stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.2.1.2 **RNA isolation using RNeasy plant mini kit**

Around 100 mg of leaf samples was ground into fine powder in liquid nitrogen and transferred into a fresh tube 2 ml centrifuge tube and added 450 µl RLT buffer, thoroughly vortexed. Transferred the lysate to spin column placed in 2 ml collection tube and centrifuged (Hemle, table top refrigerated centrifuge) for 2 min at full speed (14000 rpm). Then transferred the floe through to a new microcentrifuge tube. 0.5 volume of ethanol (96-100%) was added and mixed by pipetting. Then transferred the sample to an RNeasy spin column (pink) place in 2 ml collection tube. Followed by centrifugation for 45 sec at 8000 X g or 30 sec at 10,000 rpm and discrded the flow through. Added 700 µl buffer RW1 to the spin column and centrifuged at 10,000 rpm for 30 sec or 45 sec. After the flow through was discarded added 500 µl buffer RPE to the RNeasy spin column and centrifuged at 10,000 rpm for 15 sec. Again the flow through was discarded and added 500 µl buffer RPE and centrifuged for 2 min at 10,000 rpm. Placed the spin column in a new 2 ml collection tube and discarded the old collection tube, centrifuged at 14,000 rpm for 1 min or air dried after step 9. Placed the spin column in a new 1.5 ml tube, added 30-50 µl RNase free water, centrifuged for 2 min at 10,000 rpm. The RNA was stored at -20°c (Vest frost Low Temperature Cabinet, India).

3.3 ANALYSIS OF EXTRACTED RNA

3.3.1 Agarose gel electrophoresis

The integrity and quality of extracted RNA was checked in agarose gel electrophoresis. An agarose gel of 1.2 percent was prepared in 1X TAE buffer (Appendix 3) and 0.5 µl per litre Ethidium bromide (EtBr) was added. Three micro litre of RNA sample with the loading dye was loaded in each of the wells of the gel. The gel was run 5 V/cm for 30 min. The gel was then visualised under UV light and the image was documented using alpha imager gel documentation system.

3.3.2 Quantification of RNA

The RNA yield and purity was determined by spectrophotometric method. RNA has a great absorbance at 260 nm in UV light. RNA concentration (µg/ml) was estimated by measuring the absorbance bat 260 nm, multiplying by dilution factor, and using the relationship that an A260 of 1.0 equals 40µg/ml pure RNA. To evaluate RNA purity, the ratio of the absorbance at 260 nm by reading at 280 nm was calculated.

3.4 cDNA SYNTHESIS

3.4.1 First strand cDNA synthesis

The RNA isolated from leaf samples were subjected to cDNA conversion using M-MuLV-Reverse Transcriptsae (GeNei, Bangaluru). The components of reaction were as follows:

RNA (1000 ng/µl)	: 5 µl
10pM Oligo-dT	: 1 µl
Nuclease water	: 6 µl

The mixture was incubated at 65 °C for 5 min and chilled on ice. Followed by a brief centrifuge, the following components were added to mix.

41

10x buffer of M-MuLV : 4 µl

200µM dNTP mix : 2 µl

M-MuLV RT (200 U/µl) : 2 µl

Total volume : 20 µl

All the reagents were added, mixed by vortexing and flashed down. The cDNA conversion reaction was carried out in Eppendorf Mastercycler (Germany). The reaction conditions comprised of a reverse transcription step at 37 °C for 1 h followed by an extension step at 70 °C for 10 min

3.5 MOLECULAR DETECTION OF SPFMV

In order to detect the SPFMV virus associated with sweet potato and characterisation of identified virus through PCR, different set of primers were used and their details were provided in the table 3. These primers were synthesised from Eurofins (India). The synthesised primers (100 pM) were diluted to a final concentration of 10 pM with sterile distilled water to obtain the working solution.

3.5.1 Polymerase chain Based detection

Polymerase chain reaction (PCR) analysis was carried out with the isolated RNA using genus specific (potyvirus) and virus specific primers.

3.5.1.2 PCR analysis with potyvirus specific primers

The components of the mixture were optimised as listed below:

Water : 12.2 µl

10x Taq buffer : 2.5 µl

200µM dNTP : 1.0 µl

42

10pM Forward primer : 1.0 µl

10pM Reverse primer : 1.0 µl

Template DNA (cDNA)

(500 ng/µl) : 2.0 µl

Taq polymerase (2 U/ µl) : 0.3 µl

Total volume : 20 µl

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR programme was set with denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for MJ1/MJ2, 50.0 °C for Pot1/Hrp5 for 1 min and extension at 72°C for 1 min. Final extension was done at 72 °C for 10 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The simplified products along with PCR marker from Geni, Bangaluru were separated on agarose gel (1%) the gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

Table 4. Details of potyvirus primers used for diagnosis of potyviruses from the collected samples

Sl. No	**Primer name**	**Sequence**	**Amplified reagion**	**Amplicon size**
1	MJ1/MJ2	5'-TGGTHTGGTGYATHGARAAYGG-3' (F) 5'-TGCTGCKGCYTTCATYTG-3' (R)	WCIEN and QMKAA	327 bp
2	Pot1/Hrp-5	5'-GACTGGATCCATTBTCDATRCACC	3' end	1300,700bp

		A-3' (F) 5'-ATGATHGARKCNTGGGG-3' (R)		

3.5.1.3 PCR analysis with SPFMV1 (F)/SPFMV2 (R) primers

The components of the mixture were optimised as listed below:

Water	: 12.2 µl
10x Taq buffer	: 2.5 µl
200µM dNTP	: 1.0 µl
10pM Forward primer	: 1.0 µl
10pM Reverse primer	: 1.0 µl
Template DNA (cDNA) (500 ng/µl)	: 2.0 µl
Taq polymerase (2 U/µl)	: 0.3 µl
Total volume	: 20 µl

PCR was carried out in BioRad C100 Touch Thermocycler (Germany). PCR progamme was set with initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 93 °C 30 sec, annealing at 55.8 °C for 1 min and extension at 72 °C for 60 sec. Final extension was done at 72 °C for 7 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The simplified products along with PCR marker from Geni, Bangaluru were separated on agarose gel (1%) the gel was viewed under gel documentation system. The amplicon showing expected band size were purified by gel elution method.

Table 5. Details of SPFMV specific primers used for diagnosis of SPFMV from the collected samples

Sl. No	Primer name	Sequence	Amplified reagion	Amplicon size
1	SPFMV1/ SPFMV2	5'-ATAGTGGGGGCATCATCAAAGG-3' (F) 5'-CCTAAAAGTAGGCACTGCATGG-3' (R)	Partial Coat protein (9928-10338 bp)	411 bp

3.5.2 Analysis of amplicon by agarose gel electrophoresis

The most common method to analyse the PCR products is to run an aliquot of the sample on agarose gel. Aliquot of PCR mix (10µl) was loaded on agarose gel (1%) made of 1X TAE buffer. The gel was run 80 v/cm until the dyes migrated 3/4th of the distance through the gel. The gel was visualised and documented under the gel documentation system (Alpha Innotech) using Alpha imager software.

3.6 CHARACTERISATION OF VIRUS

3.6.1 Gel elution of PCR amplified fragments

Extraction of PCR products was carried out with QIAEX 11 gel extraction kit (QIAGEN). The PCR products were resolved on agarose gel (1%) and the amplicon was excised from the gel using a clean scalpel. The gel slice as placed into pre weighed 2 ml tube and weight was recorded. Then, add thrice the volume QX1 buffer to the gel slice. Add 30 µl of QIAEX 11 suspension and the tube was incubated at

50°C for 10 minute occasionally inverting it every 2 min to solubilise the gel. The sample was centrifuged at 13,000 rpm, 15- 25°C for 30 s and the flow through was discarded. The pellet was washed with QX1 buffer (500 µl), vortex, centrifuged for 13,000 rpm, 15-25°C and the flow through was discarded. Again the pellet was washed twice with PE buffer (500 µl), vortex, centrifuged for 30 s and the flow through was discarded. The sample tube was air dried for 30 min until the pellet become white. TE buffer (20 µl) was added to the tube, vortexed and incubated at room temperature for 5 minute. Then it was centrifuged for 30 s at 13,000 rpm, 15-25 °C band the pellet was discarded. Finally the supernatant containing the purified DNA was stored in a clean tube at -20 °C.

The gel elute was subjected to PCR using the same reaction mix under required conditions. The products of PCR were analysed using agarose gel (1%).

3.6.2 Cloning and transformation

The amplified region was cloned and transformed into *E. coli* DH5α using InsTA Clone PCR Cloning Kit (Fermentas, USA). The recombinant clones obtained were analysed by colony PCR method.

The cloning and transformation protocol was performed in three consecutive days.

Day 1:

E. coli DH5α cells revived in Luria agar medium (Appendix 5) was used for the transformation procedure. The ligation mix was prepared with the components as listed below.

Vector pTZ57R/T : 3µl

5X ligation buffer : 6 µl

PCR product : 4 µl

Nuclease free water : 16 µl

Total volume : 30 µl

After vortexing the ligation mix was centrifuged for a few seconds and incubated overnight at 4 °C. A control reaction mix was also prepared with 1 µl of control PCR fragment and incubated under similar conditions.

Day 2

To 1.5 ml C medium given in the kit (pre warmed at 37°C for at least 20 min), a portion of freshly streaked bacterial culture was transferred using inoculation loop and gently mixed to resuspended the cells. After incubating the medium at 37°C with shaking for 2 h, the bacterial cells were pelleted by 1 min centrifugation. The pellet was resuspended in 300 µl of T solution (provided in the InsTA Clone PCR Cloning Kit) and incubated for 5 min. The cells were again pelleted by centrifugation, resuspended in 120 µl of T solution and incubated on ice for 5 min. 2.5 µl of ligation mix or control mix was added to a new microfuge tubes and chilled on ice for 2 min. 50 µl of prepared cells were added to each tube containing DNA, mixed incubated on ice for 5 min and plated immediately on pre warmed LB Ampicillin X gal/IPTG agar plates (Appendix 7). The plates were incubated overnight at 37°C. Untransformed DH5α cells were plated on LB Ampicillin X gal/IPTG agar plate to serve as negative control.

Day3

The plates were observed for the presence of recombinants. The number of transformed obtained in the sample as well as positive control plate was determined.

3.6.3 Analysis of recombinant clones –colony PCR

The recombinant clones were analysed for the presence and orientation of the sequence insert by colony PCR using their respective primers (Eurofins, India). The PCR reaction mix was formulated as:

Nuclease free water	: 14.2 µl
10X Taq buffer A	: 2.5 µl
200µM dNTP	: 1.0 µl
10pM Forward primer	: 1.0 µl
10pM reverse primer	: 1.0 µl
Template DNA (colony)	: 2.0 µl
Taq polymerase (2 U/µl)	: 0.3 µl
Total volume	: 20 µl

A single white colony was selected and resuspended in the PCR reaction mix. A short strike was made over the culture plate (containing selective medium) in order to save the clone for re-propagation. The PCR was performed at the required PCR condition of each primer. The products of colony PCR were resolved on an agarose gel (1%).

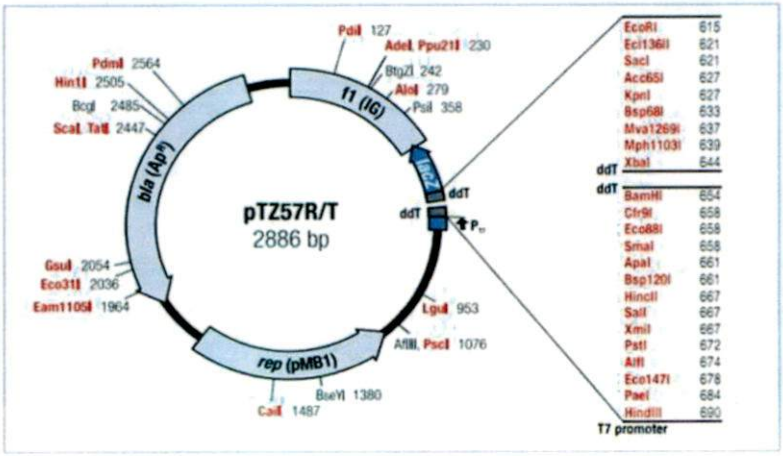


Figure 3: Vector map of pTZ57R/T

3.6.4 Plasmid isolation of transformed white colonies

Colonies which showed positive in colony PCR were selected for plasmid isolation for carrying out the downstream applications like further confirmation for the presence of inserts through restriction and sequencing.

Plasmid isolation was done using the manual method formulated as follows.

A single colony (transformed white colony) was inoculated on 2 ml LB broth with appropriate antibiotic [Here, ampicillin (50 µg/ml)] and incubated overnight with shaking of 150 rpm at 37°C. Next day, bacterial cells were pelletized by centrifuging at 10,000 rpm for 10 min at room temperature. After discarding the supernatant, the pellet was resuspended in 100 µl buffer P1 (Appendix 8) and vortexed. To this, 10 µl activated RNase (10 mg/ml) was added and incubated for 5 min at 37°C. To this 200 µl buffer P2 (Appendix 8) was added and gently mixed by inverting 6 to 7times and incubated for 5 min. To this, 150 µl P3 (Appendix 8) was added, mix gently by inverting 6 to 7 times and incubated on ice for 5 min. The mixture was centrifuged at 15,000 rpm for 30 min. The supernatant was collected and again centrifuged at 15,000 rpm for 20 min. To the collected supernantant, 0.8 volume of isopropanol was added and incubated on -20 °C for 1 hr. After incubation, the mixture was washed with 70% ethanol by centrifuging for 15,000 rpm for 15 min. After discarding the supernatant, the pellet was air dried, resuspend in sterile distilled water and stored at -20°C.

3.6.5 Confirmation of recombinant clones using restriction analysis

Restriction digestion using particular enzymes was performed for the confirmation of insert in the particular white colony

The components of the restriction mixture were optimised as listed below:

Cutsmart buffer (10 X) : 1µl

HindIII (20,000 U/ml)	: 0.5 µl
EcoR1 (20,000 U/ml)	: 0.5 µl
Water	: 6.5 µl
Plasmid DNA (150 ng/µl)	: 2.0 µl
Total volume	: 10 µl

Restriction digestion was carried out at 37°C for 25 min followed by incubation at 65 °C for 10 min for enzyme inactivation. The restricted products along with PCR marker (1kb plus) from 'Thermo Scientific" were separated on agarose gel (1%). The gel was viewed under gel documentation system.

3.6.6 DNA sequencing

Elutes of PCR products were sequenced at EUROFINS. Nucleotide BLAST of the obtained sequence was performed in order to find out the similar sequences.

3.6.7 Sequence analysis

The Electrophoregram obtained by the sequencing was analysed and blasted using the basic alignment tools (BLASTn and BLASTx) in the NCBI website. The sequences having more than 80% identity were selected from NCBI and multiple sequence alignment was done. Phlogenetic tree was constructed from Bio Edit aligned sequences using MEGA 7.0.26 analysis tool.

RESULTS

4. RESULTS

The results of the study entitled "Molecular characterization of *Sweet potato feathery mottle virus*" conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017 are presented in this chapter.

4.1 COLLECTION OF SAMPLES AND SYMPTOMATOLOGY STUDIES

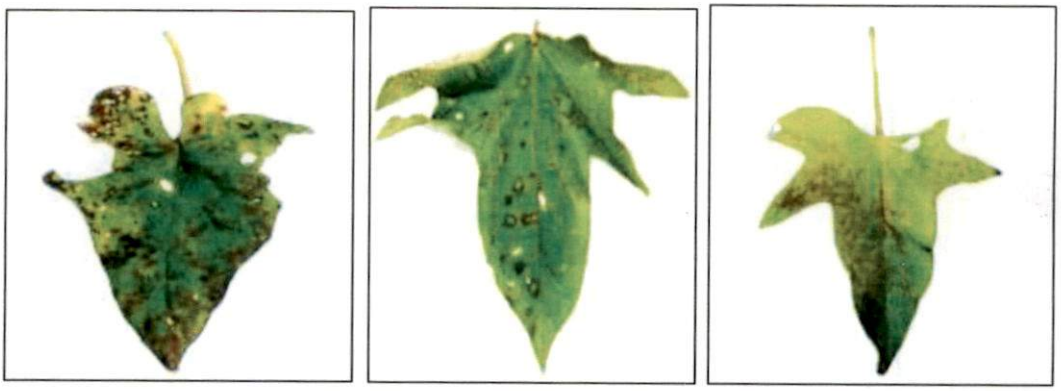
Sweet potato samples belonging to various accessions were collected randomly from the germplasm collection maintained at ICAR-CTCRI, Thiruvananthapuram. Leaf samples from 45 accessions exhibiting viral symptoms were collected, serially labeled and symptomatology was recorded by visual observation. The sample details and symptoms observed are represented in Table 6 and representative samples are shown in Figure 4. This representative sample set was used for further tests and analysis.

4.2 NUCLEIC ACID EXTRACTION

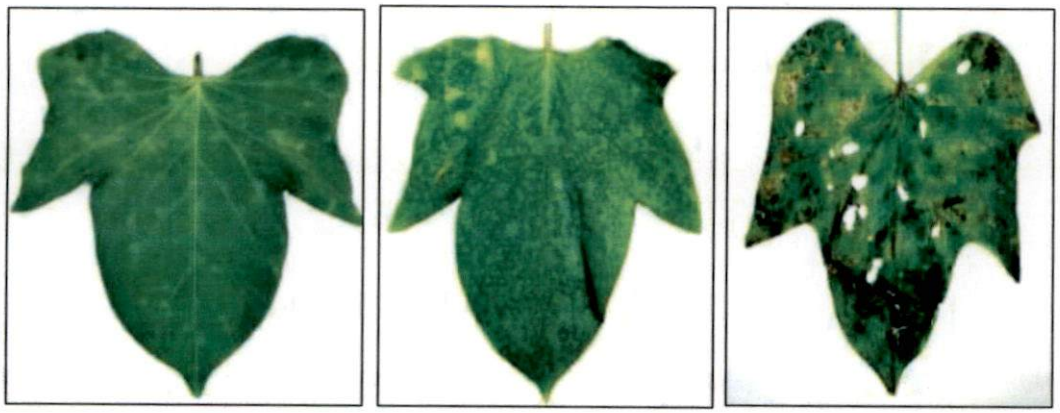
4.2.1 Isolation of total RNA

Total RNA isolation from representative 45 samples were carried out using TRIzol reagent and $LiCl_2$ protocol. The extracted RNA was run on an agarose gel (1.2%) and visualised under UV to observe the bands. Comparatively good quality RNA is obtained with second protocol ($LiCl_2$). To quantify the isolated RNA, spectrophotometric readings at 260 nm (OD_{260}) were taken and noted the quantity in 1 µg/ml. Purity of isolated RNA was calculated using OD_{260} and OD_{280}. Amount of isolated RNA from 45 accessions varied between 50µg/ml and 300µg/ml and the purity varried in the range of 1.80 to 2.40. The gel image showing RNA obtained as bright bands are shown in page 34-35.

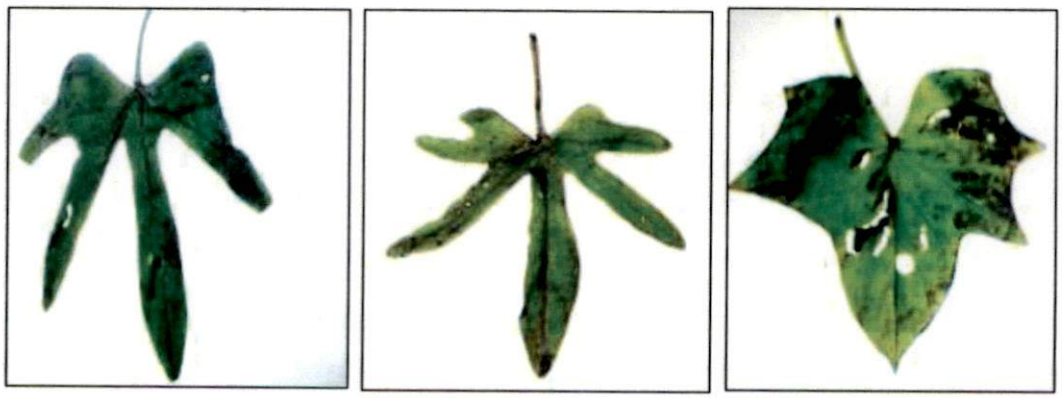
Figure 4: Representative sample set (symptoms detail in table 4)



Sample1 Sample 2 Sample 3



Sample 4 Sample 5 Sample 6



Sample 7 Sample 8 Sample 9

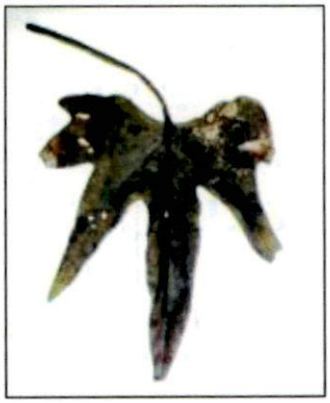
Sample 10

Sample 11

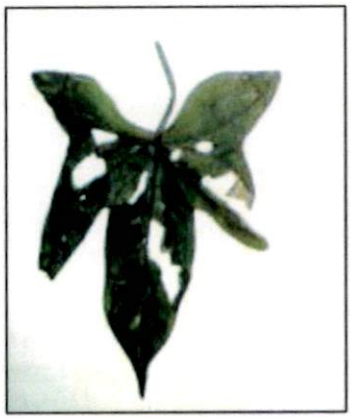
Sample 12

Sample 13

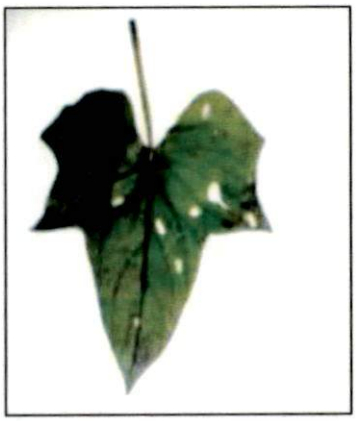
Sample 14



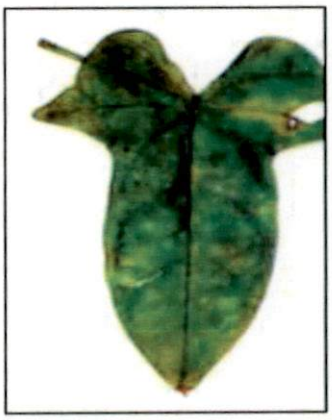
Sample 15



Sample 16



Sample 17



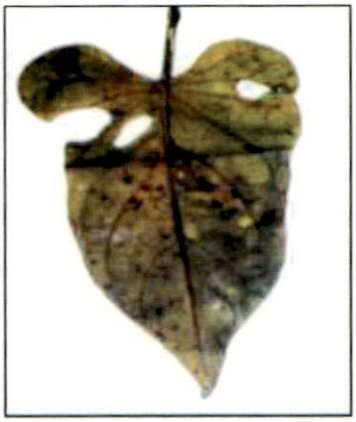
Sample 18

Sample 19

Sample 20

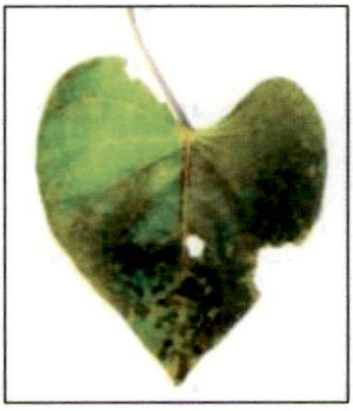
Sample 21

Sample 22

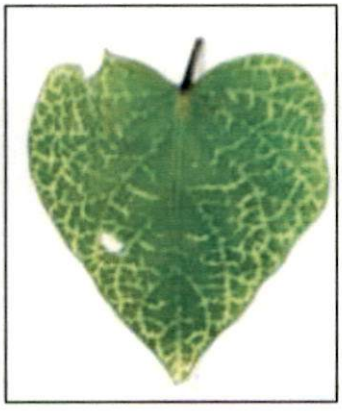


Sample 23

Sample 24

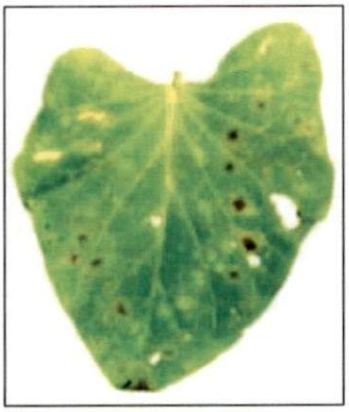


Sample 25



Sample 26

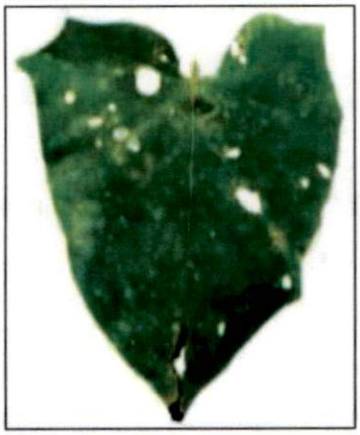
Sample 27



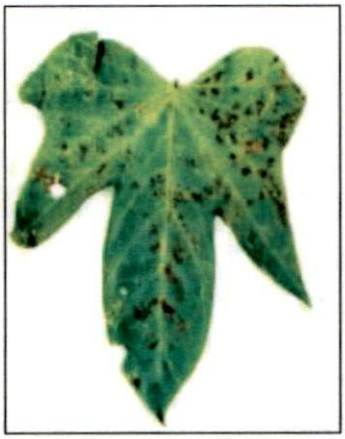
Sample 28

Sample 29

Sample 30



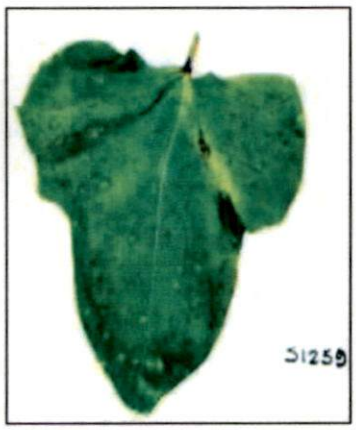
Sample 31



Sample 32

Sample 33

Sample 34

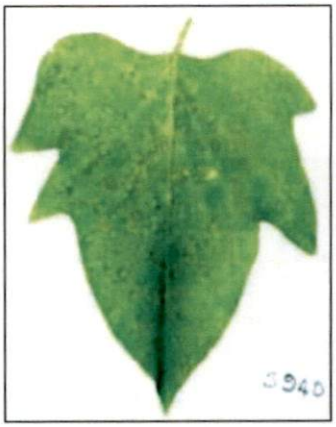


Sample 35

Sample 36

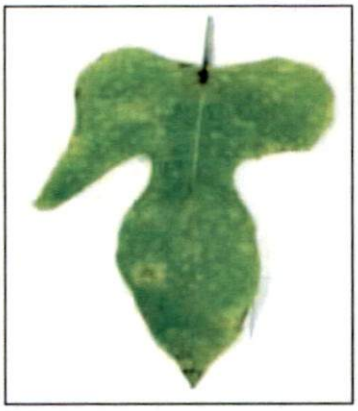
Sample 37

Sample 38

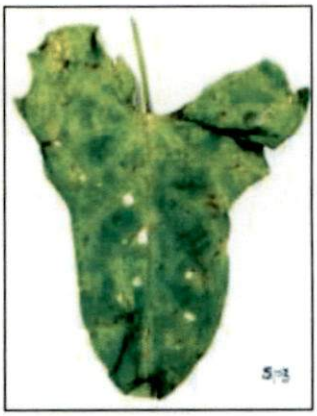


Sample 39

Sample 40

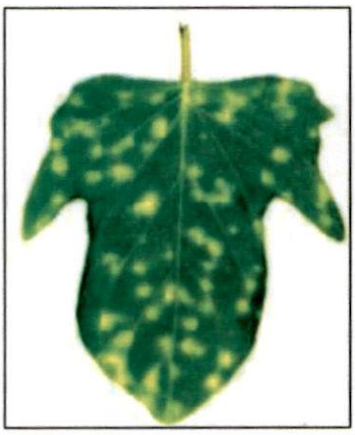


Sample 41

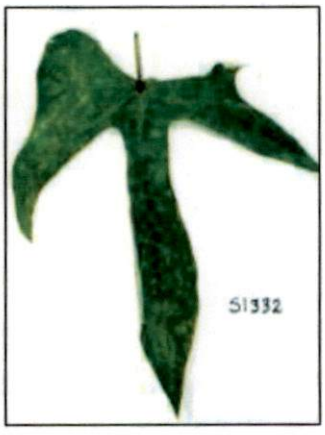


Sample 42

Sample 43



Sample 44



Sample 45

Table 6: Reprsentative sample set, symtoms observed

Sample No	Accession ID	Symptoms
1	S1360	Feathering
2	Sree Bhadra	Purple ring spots
3	S1312	Feathering
4	S710	Chlorotic spots
5	S700	Dark green spots with feathering
6	Sree Varun	Ring spot with feathering
7	S188	Chlorotic spots
8	S658	Chlorosis
9	S1163	Ring spot with feathering
10	S758	Marginal chlorosis
11	S420053	Ringspots with feathring
12	S1203	Marginal chlorosis
13	S1294	Vein clearing
14	S390	Chlorotic spots
15	S420365	Feathering
16	S757	Purple Ringspot
17	S1231	Feathering
18	S275	Chlorotic spots
19	S732	Feathering
20	S219	Feathring

Sample No	Accession ID	Symptoms
21	S270	Purple Ring spots
22	S25	Chlorotic spots
23	S18	Purple Ring spots
24	S632	Purple Ringspot with feathering
25	S44013	Feathering
26	S1364	Yellow netting
27	Kanjagad	Purple ring spots
28	Sree Arun	Vein clearing and purple ring spots
29	Kanaka	Purple ring spots
30	SD15	Chlorotic spots with purple borders
31	Sree Nandhini	Chlorotic spots
32	Sree Rethna	Purple sopts with vein clearing
33	Sree Vardhini	Purple ring spots
34	Sourin	Feathering
35	S1259	Chlorotic spots with out purple borders
36	SH171	Curling with Feathering
37	S971	Chlorotic spots with purple borders
38	S112/11	Curling with light vein clearing
39	S940	Chlorotic spots with purple borders
40	S1323	Purple ring spots
41	S1026	Marinal chlorosis
42	SP3	Curling with veinal chlorosis

Sample No	Accession ID	Symptoms
43	S227	Yellow netting
44	Shanghar	Chlorosis
45	S 1332	Feathering

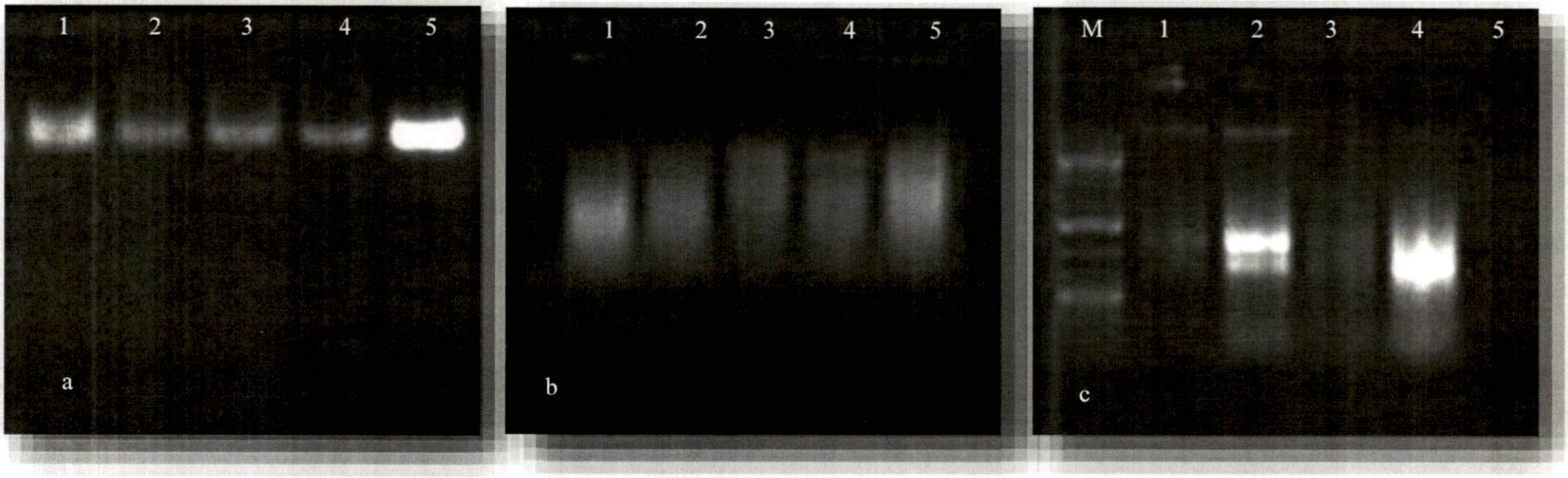


Figure 5: Total RNA isolation a. By TRIzol method, b. By Lithium Chloride, c. RNeasy plant mini kit
Lanes: 1= S1360, 2=Sree Bhadra, 3=S1312, 4=S710, 5=S700
Marker:1kb plus ladder

Table 7: Quantitative and quantitative comparison of three methods

Accession ID	**Methods of RNA isolaton**					
	TRIzol method		**Lithium chloride method**		**By RNeasy plant mini kit method**	
	Amt. of RNA (µg/ml)	**Purity (A_{260}/A_{280})**	**Amt. of RNA (µg/ml)**	**Purity (A_{260}/A_{280})**	**Amt. of RNA (µg/ml)**	**Purity (A_{260}/A_{280})**
S1360	507.7	1.39	166.4	2.31	369.35	2.21
Sree Bhadra	628.0	1.87	99.50	2.34	543.64	2.20
S1312	571.0	1.24	187.8	2.27	451.20	2.27
S710	714.0	1.54	269.0	2.23	396.32	2.23
S700	562.0	1.40	129.0	2.00	345.5	2.10

62

Table 8: Quantification of total RNA of representative set (Lithium chloride method)

Sample No	Accession ID	Amt. Of RNA (µg/ml)	Purity (A_{260}/A_{280})
1	S1360	166.4	2.31
2	Sree Bhadra	99.50	2.34
3	S1312	187.8	2.27
4	S710	269.0	2.23
5	S700	129.0	2.00
6	Sree Varun	145.2	2.15
7	S188	156.3	2.35
8	S658	116.3	2.53
9	S1163	134.0	2.45
10	S758	168.0	2.43
11	S420053	105.2	2.18
12	S1203	114.3	1.89
13	S1294	176.3	2.47
14	S390	203.0	2.33
15	S420365	235.5	2.21
16	S757	221.7	2.36
17	S1231	261.6	2.31
18	S275	146.1	2.28
19	S732	110.9	2.11
20	S219	153.30	2.36
21	S270	252.4	1.91

Sample No	Accession ID	Amt. Of RNA (µg/ml)	Purity (A_{260}/A_{280})
22	S25	143.6	2.3
23	S18	267.4	2.32
24	S632	220.0	1.99
25	S44013	124.2	2.07
26	S1364	260.8	2.21
27	S832	178.1	2.0
28	Sree Arun	235.5	2.3
29	SCO3	156.8	2.22
30	SD15	109.3	2.12
31	Sree Nandhini	220.7	1.9
32	Sree Rethna	309.8	2.34
33	Sree Vardhini	167.5	2.17
34	Sourin	260.0	2.09
35	S1259	223.6	2.26
36	SH171	187.4	1.99
37	S971	212.4	2.0
38	S112/11	134.6	2.22
39	S940	184.5	2.31
40	S1323	245.0	2.25
41	S1026	165.4	2.1
42	SP3	230.0	2.3

64

Sample No	Accession ID	Amt. Of RNA (µg/ml)	Purity (A_{260}/A_{280})
43	S227	350.6	2.21
44	Shanghar	178.4	2.26
45	1332	285.0	2.32

4.2.2 cDNA synthesis

The RNA isolated from the 45 accessions were converted into cDNA for further PCR based screening. As isolated RNA is not stable for long term storage, it was converted into cDNA which ensured the availability of sample for further tests. cDNA conversion was carried out using M-MuLV Reverse Transcriptase and oligo-dT primer. The synthesised cDNA was run on an agarose gel (1%) and visualised under UV to observe the bright sheared bands.

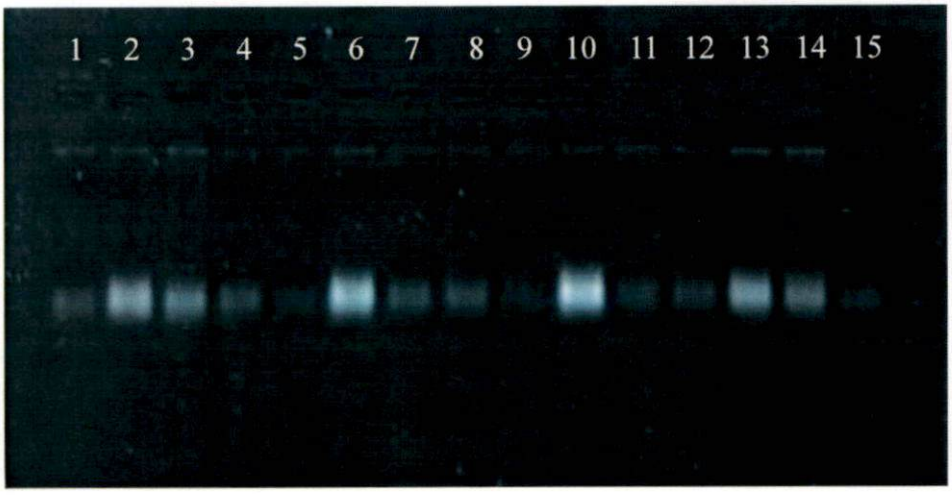


Figure 6: Gel profile of cDNA

Lanes: 1=S700, 2=SH710, 3=S1294, 4=S275, 5= S940 6=S658, 7=S188, 8=S1360, 9= S1163, 10=S758, 11=S420053, 12=S1203, 13= S390, 14=Sree Arun

4.3 MOLECULAR DETECTION OF VIRUSES

Polymerase chain reaction was employed as a part of molecular detection and diagnosis. Group specific and virus specific primers were used for the detection of major virus SPFMV infecting sweet potato. Primers were got synthesised from Eurofins, India.

4.3.1 PCR for detection of potyviruses

Detection of potyvirus infection was carried out using potyvirus group specific primers (Table 4) providing corresponding PCR conditions. A non-template was used as control having all components of a typical PCR but no template DNA. PCR was done for the representative 45 samples for detection of potyviruses. Single band representing the amplicon size 327 for MJ1/MJ2 and 700 for Pot1/Hrp5 were observed for virus positive samples in agarose gel (1%) electrophoresis. No amplification was observed in non-template control, which indicates there is no non-specific binding in PCR. Gel electrophoresis images are shown in Figure 7 and 8.

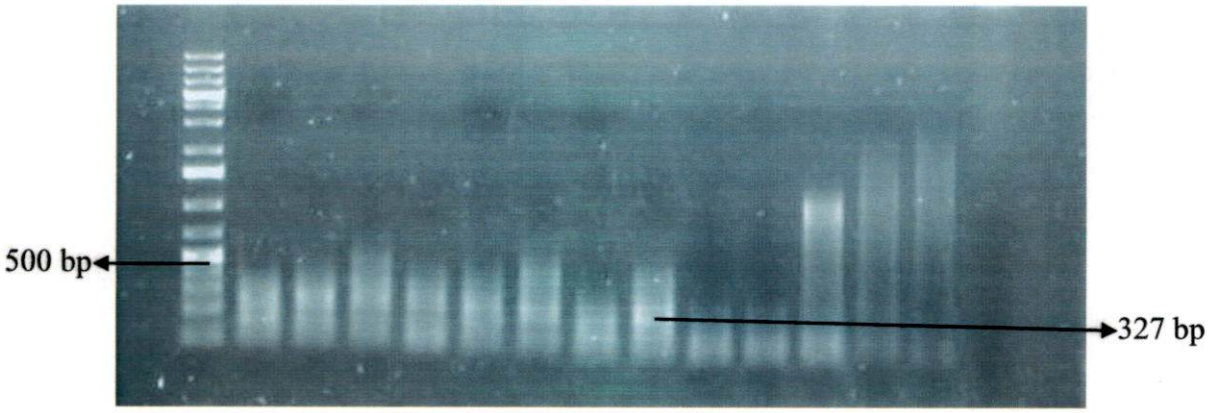


Figure 7: Sample showing positive for MJ1/MJ2

M=1kb plus ladder

Lanes: 1=S20053, 2=S188, 3=S658, 4=SH710, 5=S1294, 6=S275, 7=S940, 8=S78, 9=S18, 10=S971, 11= S420053, 12= S1203 13= S390, 14= Sree Arun, 15= S757

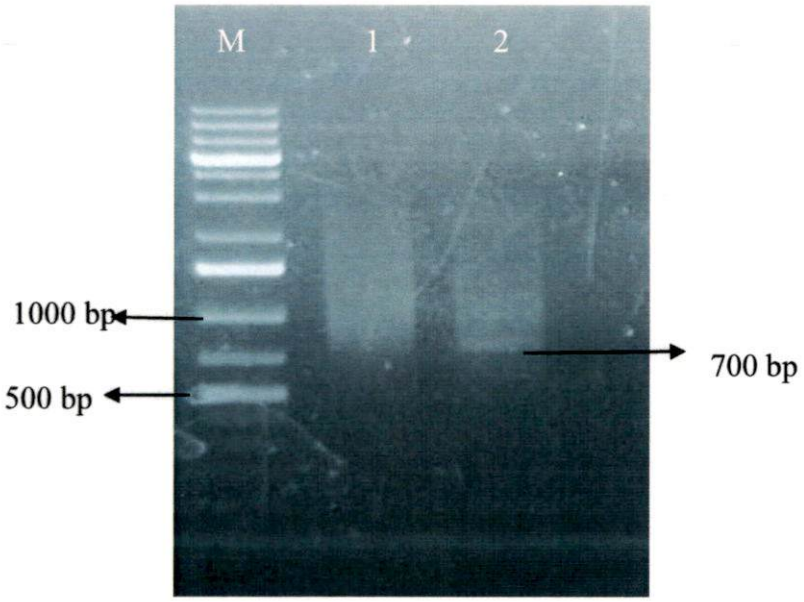


Figure 8: Sample showing positive for Pot1/Hrp5

M=1kb plus ladder,

Lanes: 1=S1360, 2=SH710

4.3.2 PCR analysis with virus specific primers

Virus specific primers are used for the detection of severely infecting viruses in sweet potato. These primers code for the specific region in the genome of respective virus

4.3.2.1 PCR detection of SPFMV

Detection of SPFMV infection in representative samples were carried out using SPFMV1 and SPFMV2 specific primers which amplified the partial Coat protein gene and gave amplified product of 411 bp. A non-template was used as control having all the components of a typical PCR but no template DNA. PCR conditions were mentioned in 3.5.1.3. An amplification size 411 bp was observed as a single band for 5 virus infected samples in agarose gel (1%) electrophoresis. No amplification was observed in the non-template control, which indicates there is no non-specific binding in PCR.

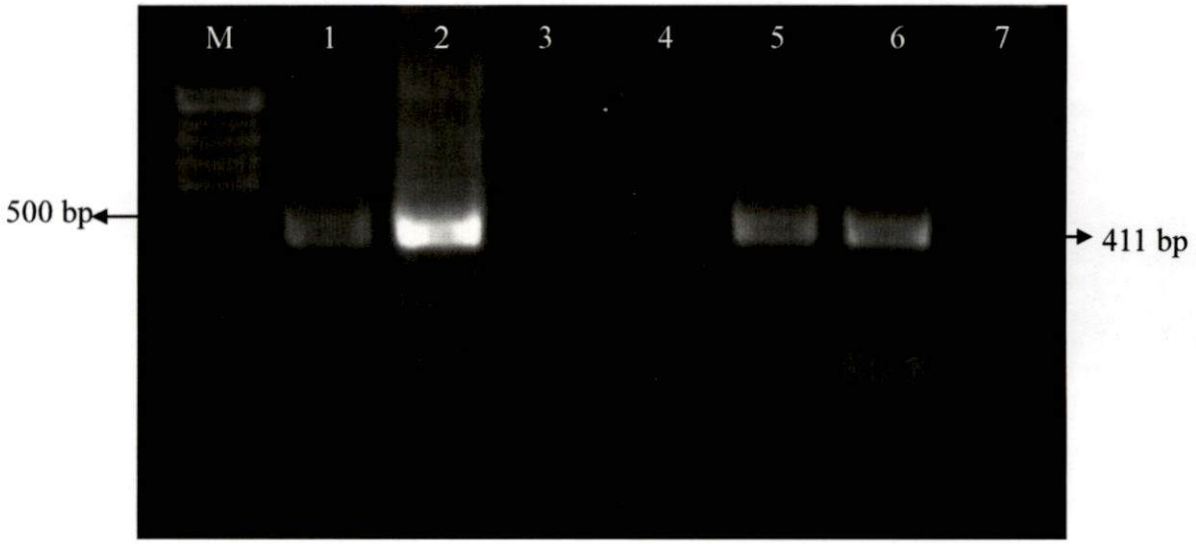


Figure 9: Sample showing SPFMV positive for SPFMV1 (F)/SPFMV2 (R)

M=100 bp ladder

Lanes: 1=S700, 2=SH710, 3=S188, 4=S658, 5=S1294, 6=S275, 7=S940

4.4 CHARACTERISATION OF THE VIRUSES

Cloning and transformation was employed as a part of molecular characterization of virus and further sequence analysis is done for further confirmation.

4.4.1 Cloning and transformation

The gel elutes of 411bp & 700bp from SH710 were proceeded with cloning for sequencing analysis. The eluted amplified products where cloned into pTZ57R/T vector using InsTA clone PCR Cloning Kit (Fermentas, USA) and transformation was carried out in *E.coli* DH5α. The transformed colonies were distinguished by

blue-white screening in LB Ampicillin X gal/IPTG (Figure 10). Around 25-50 colonies were obtained in each plate. Grid plates were prepared to maintain the transformed white colonies. The white colonies were selected for further analysis using colony PCR (Figure 10). The recombinant clones were analysed by colony PCR using respective primer set under appropriate conditions and they were analysed in 1% agarose gel. Out of the 19 colonies 10 of them showed positive. Plasmid DNA was isolated from these positive clones. The inserted gene of interest was confirmed by restriction with *(EcoR1* and *Hind111*) release of the isolated plasmids using restriction enzymes. The 9 positive clones were sent to Eurofins for DNA sequencing, with their primers. The sequencing results were obtained as electrophoregram.

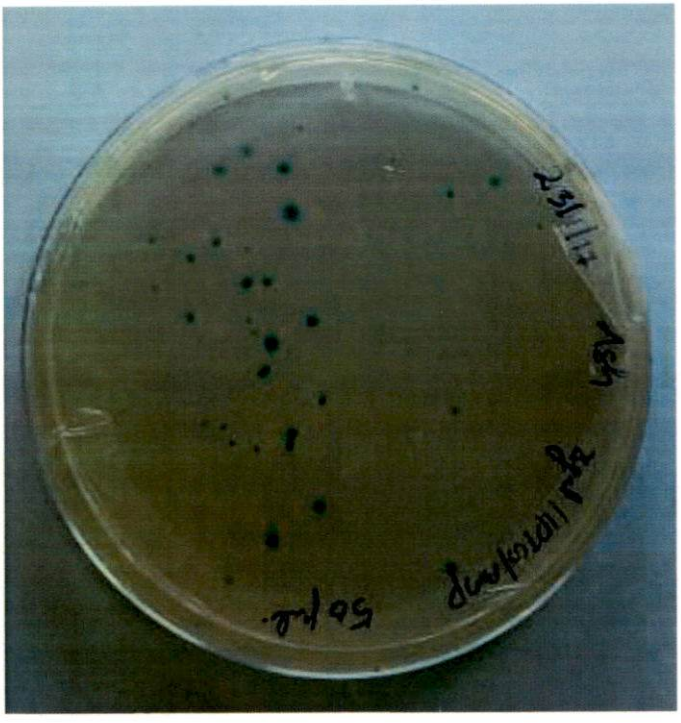


Figure 10: Colonies obtained for transformation using PCR product of partial CP in LB-AXI plates

Insert: Partial CP, Vector: pTZ57R/T, Host: *E.Coli* DH5α

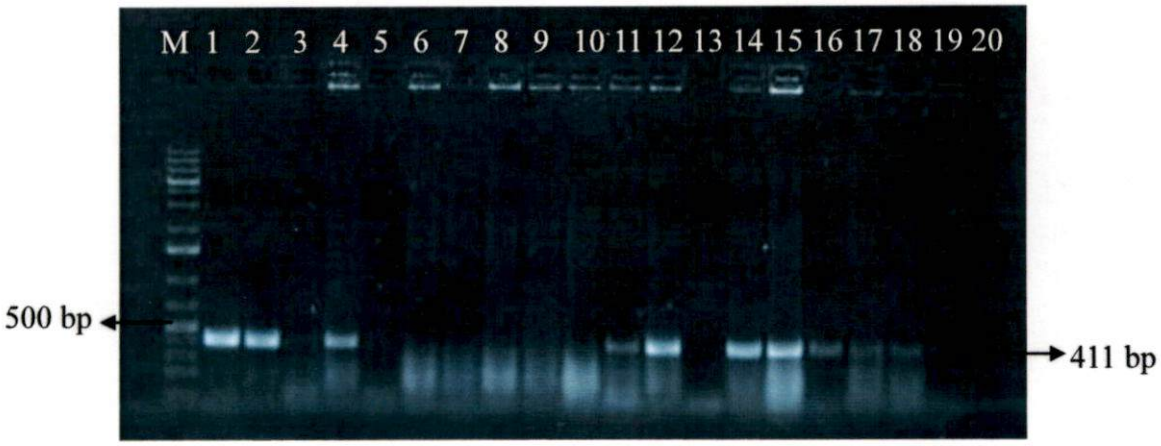


Figure 11: Colony PCR to confirm insertion of partial CP amplicon

M=1kb plus ladder

Lane 1 to Lane 19= Clone1 to Clone 19

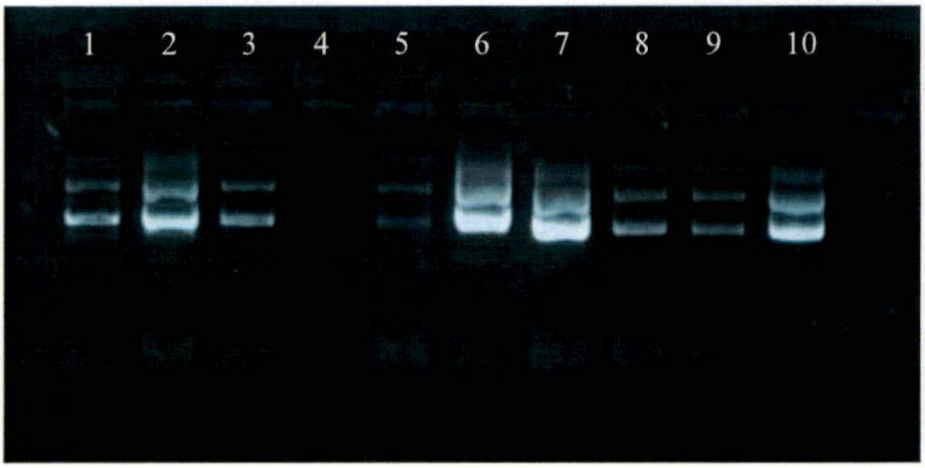


Figure 12: Isolated positive *E.coli* DH5α plasmid clones

Lane 1 to Lane 10= Plasmid from 10 clones

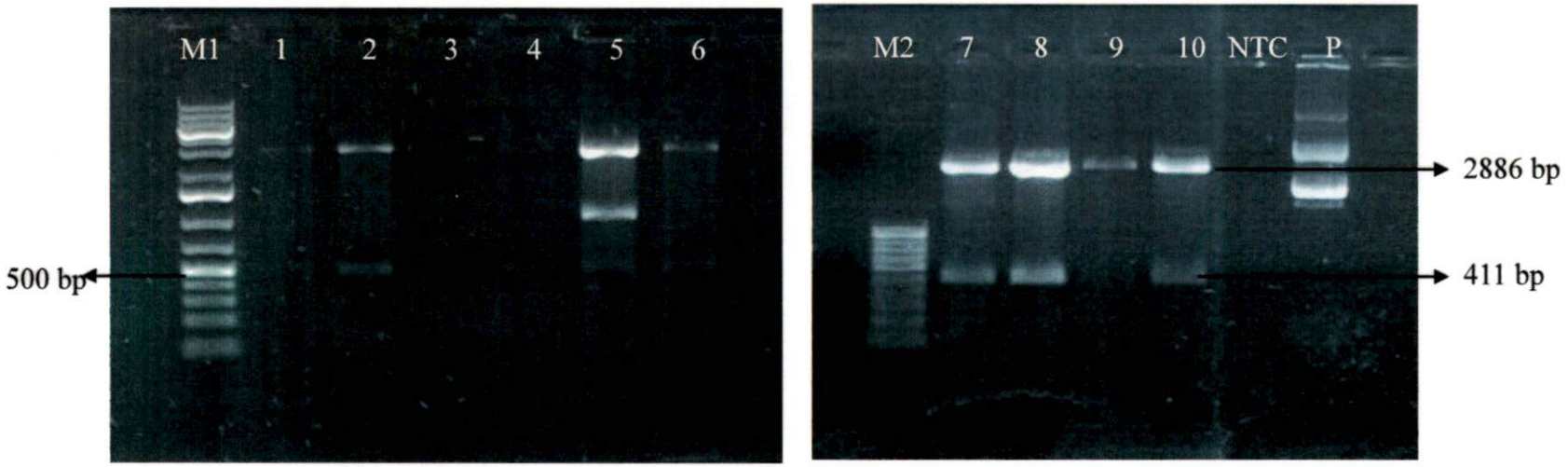


Figure13: Restriction digestion of *E.coli* plasmid

M1=1kb plus ladder
M2=100bp ladder
NTC-=Non template control
P=Non restricted Plasmid
Restriction enzymes = *EcoRI* and *HindIII*

4.5 SEQUENCE ANALYSIS

4.5.1 Sequence analysis of PCR products

The 9 positive plasmid clones were sent to Eurofins for DNA sequencing, with the SPFMV1 (F)/SPFMV2 (R) primers. The sequencing results were obtained as electrophoregram. From the sequencing results, it was found that totally 8 clones shown variation which comprises, two Korian isolate and five Argentina and one Chineese isolate. These sequences were used for further analysis and are listed below.

Table 9: NCBI BLAST results of inserts sequences

Clone No	**Insert length (bp)**	**NCBI Hit**	**E value**	**Maximum score**	**Percentage identity**
1	407	*Sweet potato feathery mottle virus* isolate CW137, complete genome	0.0	684	98%
2	408	*Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene, complete cds	0.0	704	98%
3	407	*Sweet potato feathery mottle virus* isolate CW137, complete genome	0.0	682	98%
4	408	*Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene, complete cds	0.0	708	98%

5	408	*Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene, complete cds	0.0	708	98%
6	332	*Sweet potato feathery mottle virus* isolate Shaanxi2 coat protein gene, partial cds	4e-118	417	88%
7	408	*Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene, complete cds	0.0	708	98%
8	408	*Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene, complete cds	0.0	708	98%

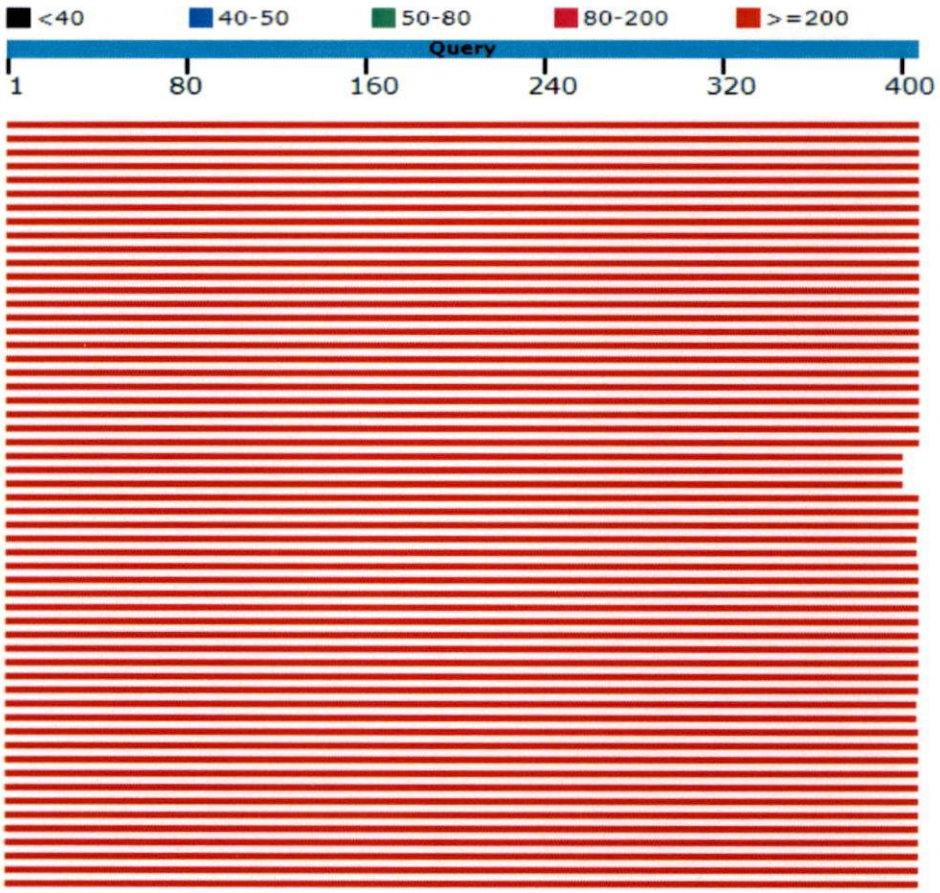


Figure 14: BLAST analysis results for clone 2, 4, 5, 7, 8

Our sequence has 99% identity and 98% query coverage with Argentina isolate SPFMV-O-Arg polyprotein gene. The sequence aligned with the coat protein region between 9921bp and 10321bp with five mismatches. This is categorized as "Ordinary" strain. The sequence obtained is given below (408 nts):

>SPFMV_A

CCTAAAAGTAGGCACTGCATGATCCAATAACGGTTTAATTGGGTATGT
CACTTGCTCATCGCCGTCCATCATTGTCCACACACCATTTATATTTGGG
GATGTGCCATTTTCAATGCACCAAACCATTAGTCCATTCATTAAGATCC
CCATTCCTGTGTCGTCAACACCGTAATCCCCTTTAACTCCCTCATACCA
TGCTTGGAATTGCTCTTGGGTCGAACGGGTGTTCGCAACTTCATACTGC

TCTGGTTCATATGTTGACAAGTGTTGGAAATTTATAATGGCCCTTCCAT
TGACCATTGGTTGTCTCTTCTTATTTGCATTCATCTTAACACGTGGTAC
AACAAATGTACCAACTGTGCCAACATTAACGTCTTTATCTCTCACACCC
TTTGATGATGCCACTAT

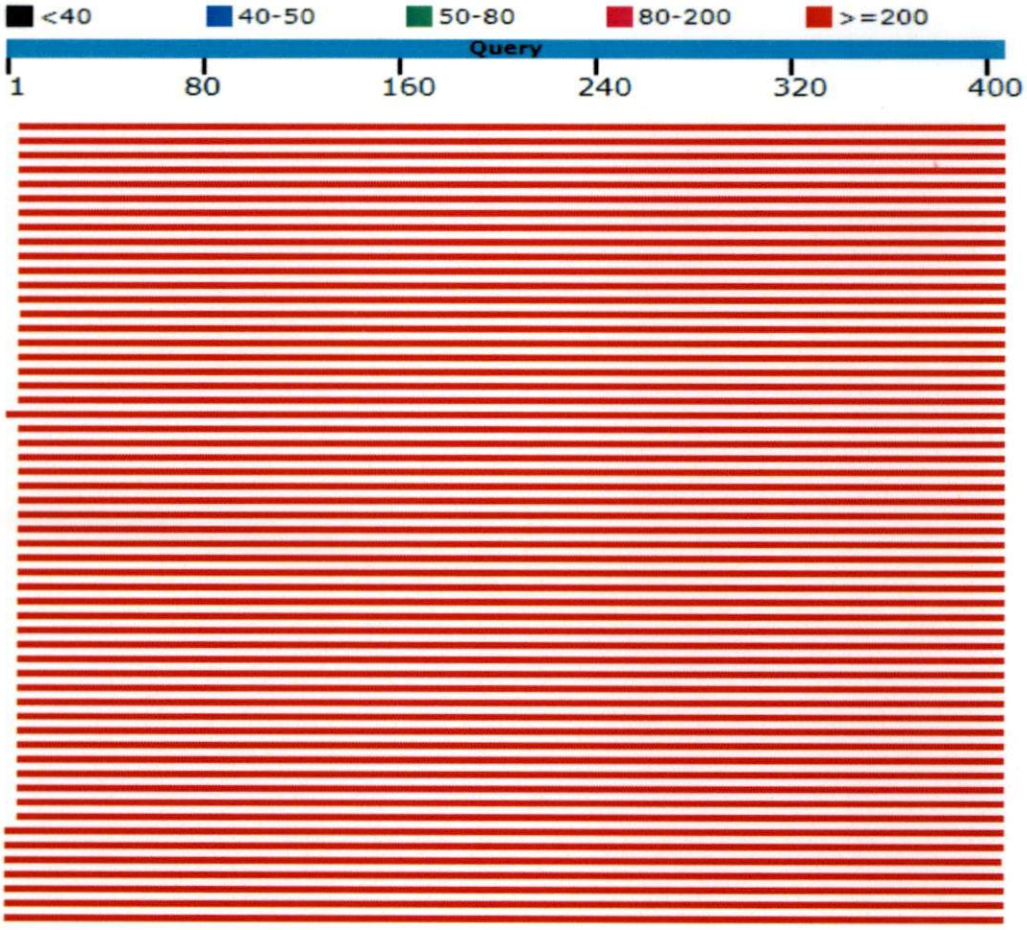


Figure 15: BLAST analysis result for clone 1, 3

Our sequence has 98% identity and 98% query coverage with Korean isolate named CW137. The sequence aligned with the coat protein region between 9860 bp and 10261 bp with seven mismatches. Based on phylogenetic analyses and sequence comparisons, the Korean SPFMV isolates belonged to the strains RC and O with >98% nucleotide sequence identity. The sequence obtained is given below (407 nts):

>SPFMV_B

ATAGTGGCATCATCAAAGGGTTTGAGGGATAAAGATGTAAACGTTGGT
ACAGTTGGCACGTTTGTCGTGCCACGTGTTAAGATGAATGCAAACAAG
AAAAGGCAACCAATGGTAAATGGAAGGGCCATTATAAATTTCCAACA
CTTGTCAATATATGAGCCAGAACAGTTTGAGGCTGCAAACACCCGGTC
GACTCAAGAACAGTTTCAAGCATGGTATGAGGGAGTGAAAGGAGACT
ATGGTGTTGATGACGCAGGAATGGGGATTTTATTGAATGGATTAATGG
TTTGGTGCATTGAAAATGGCACATCCCCAAATATAAATGGTGTGTGGA
CTATGATGGATGGTGATGAGCAAGTGACATATCCAATTAAACCATTGT
TGGACCATGCAGTGCCTACTTTTAG

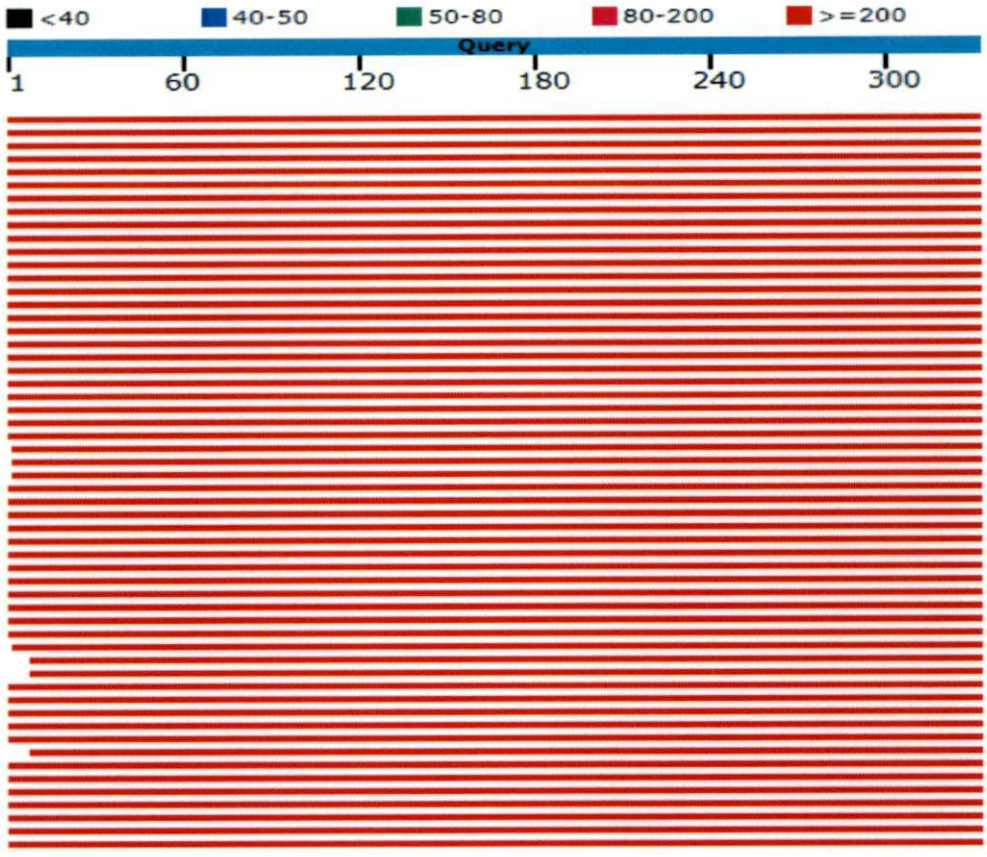


Figure 16: BLAST analysis result for clone 6

Our sequence has 91% similarity and 92% query coverage with Chinese isolate named Shaanix2. The sequence aligned with the coat protein region between 212 bp and 515 bp with three deletions. Chinese isolate in turns shows similarity with

212 bp and 515 bp with three deletions. Chineese isolate in turns shows similarity with "Ordinary", "Russet crack" and "East African" strain Ordinary. The sequence obtained is given below (305 nts):

>SPFMV_C

CATCATCAAAGGGTGTGAGAGATAAAGACGTTAATGTTGGCACAGTTGGT
ACATTTGTTGTACCACGTGTTAAGATGAATGCAAATAAGAAGAGACAACC
AATGGTCAATGGAAGGGCCATTATAAATTTCCAACACTTGTCAACATATG
AACCAGAGCAGTATGAAGTTGCGAACACCCGTTCGACCCAAGAGCAATTC
CAAGCATGGTATGAGGGAGTTAAAGGGGATTACGGTGTTGACGACACAG
GAATGGGGATTTTATTGAATGGACTAATGGTTTGGTGCATTGAAAATGGC
ACATC

4.5.2 Construction of phylogenetic tree

Using 13 reference sequences (Table 10) and 3 sample sequences from this study, multiple alignments was done in CLUSTALW program and generated a phylogenetic tree using UPMA and neighbor joining option in MEGA 7.0.26. Query sequence 1 (SPFMV_A), sequence 2 (SPFMV_B) and sequence 3 (SPFMV_C) form different clusters.

Table. 10: Reference sequences used for phylogenetic tree construction

NCBI ID	**Description**
KP115608.1	*Sweet potato feathery mottle virus* isolate CW137, complete genome
KC771329.1	*Sweet potato feathery mottle virus* isolate SC10 coat protein gene, partial cds
KC771330.1	*Sweet potato feathery mottle virus* isolate SC11 coat protein gene, partial cds

HQ844123.1	*Sweet potato feathery mottle virus* isolate Shaanxi2 coat protein gene, partial cds
HQ844098.1	*Sweet potato feathery mottle virus* isolate Hunan3 coat protein gene, partial cds
KF386013.1	*Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene, complete cds
AB465608.1	*Sweet potato feathery mottle virus* genomic RNA, complete genome, strain: ordinary
D16664.1	*Sweet potato feathery mottle virus* gene for polyprotein, partial cds
KP115609.1	*Sweet potato feathery mottle virus* isolate GJ122, complete genome
KC771331.1	*Sweet potato feathery mottle virus* isolate SC12 coat protein gene, partial cds
FJ867906.1	*Sweet potato feathery mottle virus* isolate IS8 polyprotein gene, partial cds
KF386014.1	*Sweet potato feathery mottle virus* isolate SPFMV-RC-ARg polyprotein gene, complete cds
HQ844117.1	*Sweet potato feathery mottle virus* isolate Shanxi2 coat protein gene, partial cds
And query sequences 1,2&3	SPFMV_A SPFMV_B SPFMV_C

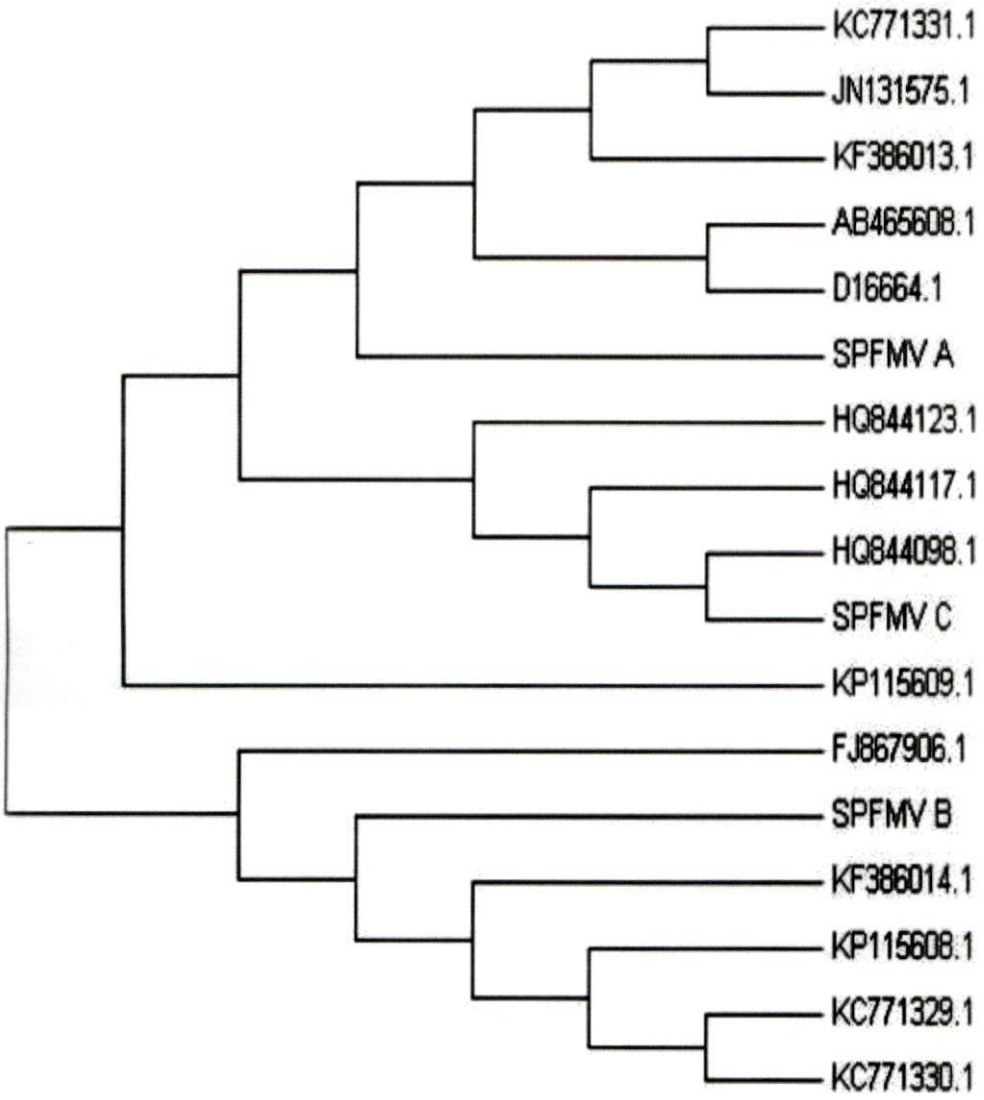


Fig. 18: Phylogenetic tree of the sequenced samples obtained using MEGA 7.0.26.

DISCUSSION

5. DISCUSSION

Tropical root and tuber crops (sweet potato, cassava, aroids and yams) are the third most important food crops in the world after cereals and legumes making significant contributions to household food security, sustainable development and income generation in developing countries (Hegde *et al.,* 2008).

Sweet potato (*Ipomoea batatas* L. Lam) is a tropical plant which is cultivated for its tubers and leaves, belonging to the family convolvulaceae. It is considered as "food for poor" by certain group of population as it is generally associated with scarcity periods (Yao *et al.*, 2005)

Sweet potato cultivation is generally constrained, especially by viral disease that cause over 50% of yield reduction (Mukasa *et al.*, 2006). Viruses build up from generation to generation as a result of vegetative propagation (Okpul *et al.*, 2011). Sweet potato viruses are mainly spread through vines that looks healthy, which are collected from the previous crops for the next section of cropping. Thus singly infected virus can act as a source of inoculum and through vector transmission lead to mixed infections of different viruses (Rukaewa *et al.*, 2010).

More than 30 viruses have now been reported to infect sweet potato (Clark *et al.*, 2012). The five main viruses identified to infect sweet potato in India are *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV) , *Sweet potato latent virus* (SPLV), *Sweet potato chlorotic fleck virus* (SPDFV) and *Sweet potato leaf curl virus (*SPLCV) (Makeshkumar *et al.,* 2001).

Multiple virus infection in sweet potato are a common phenomenan (Karyeija et al., 2000). The major one is the SPFMV, component of SPVD combined with the SPCSV (Brunt *et al.*, 1996). SPFMV has long been observed to occur wherever sweet potatoes are grown. SPMMV has occurred most frequently in mixed infectios with

SPCSV (Mukasa *et al.*, 2003). SPMMV has also occurred in complex with SPCSV and SPFMV (Mukasa *et al.,* 2004).

Four closely related potyviruses, including SPFMV, Sweet potato virus 2 (SPV2), Sweet potato virus C (SPVC), and Sweet potato virus G (SPVG) are important in sweet potato virus disease complexes all over. Identification and detection of these viruses are confusing by the frequent occurrence as mixed infections and low titer in many sweet potato cultivares and high similarity among their genomic sequences. A one-tube multiplex RT-PCR (mRT-PCR) assay was used for simultaneous identification and differentiation of SPFMV, SPV2, SPVC and SPVG (Abad *et al.*, 2012).

SPFMV, a type member of potyvirus, is transmitted by several genera of aphids in non persistant manner (Berger *et al.*, 2005). Symptoms changes with regared to the cultivated variety and often identification of virus becomes difficult due to environmental factors. SPFMV consists of four strains: common (C), East-African (EA), ordinary (O), and russet cracks (RC) which are readily distinguished by phylogenetic analysis of the CP encoding sequences (Kreuze *et al.*, 2000). Tairo *et al.*, (2005) reported that Strain C is phylogenetically distant from the other three strains and suggested a separate classification of the virus.

In India, presence of SPFMV and SPLCGV co-infection was first reported by Prasanth and Hedge in 2008. In Kerala also it is the first report. SPFMV is also reported in Andra Pradesh, Odisha (Prasanth & Hegde, 2008) and West Bangal (Sinha & Tarafdar, 2007).

For the detection of SPFMV, serological methods like NCM-ELISA, and DAC-ELISA are widely used. But its low sensitivity during periods of low titre is a major limitation. Because of frequent serological cross-reaction between species of

potyviruses serological diagnosis is often not impressive and biological indexing is also cumbersome (Brunt *et al.*, 1992).

Electron microscopy studies revealed that SPFMV is a potyvirus with average length of 810 nm.The virus was purified from SPFMV infected sweet potato leaves. The antiserum was produced and tested using Ouchterlony double-diffusion test. SPFMV was detected in different samples using direct antigen coating ELISA and nitrocellulose membrane ELISA (Jeeva *et al.,* 2004)

Nucleic acid spot hybridization using riboprobe which is highly sensitive and can detect up to 0.128 pg of RNA has also been developed (Abad and Moyer, 1992). Hegde *et al.* (2007b) developed non radioactive biotinylated probe using the coat protein gene of the SPFMV Trivandrum isolate in India for the detection of SPFMV.

In this study, the virus SPFMV was screened for its presence in sweet potato through nucleic acid based method of virus detecton. Following the symptomatology study, samples were subjected to PCR based virus detection using group specific and virus specific primers using RT-PCR method. SPFMV Symptoms on the foliage are generally more prevalent on older leaves. The irregular classical patterns of chlorosis known as feathering along veins of leaves and distinct-to-faint chlorotic spots having no margins to margins of purple colour and on young leaves in some cultivar mild mottle also occur (Makeshkumar et al., 2001; Jeeva et al., 2004).

The PCR based methods are rapid, highly sensitive and useful for accurate detection, quantification and charecterisation of plant pathogens. Among the 45 accessions selected, two pairs of potyvirus specific primers used in this study showed that 10 of them gave amplicon of 327 bp for MJ1/MJ2 primers and 1 sample gave amplicon of 700 bp for Pot1/Hrp5. Other potyvirus group specific primers like Atropa Nad2. 1a/Atropa Nad2. 2b amplifying NAD region, Nib2F/Nib3R amplifying Nib region Hrp-5/oligo-dT amplifying 3' end gave amplicons of 188 bp/800 bp, 350 bp and 1500 bp respectively.

Out of 45 accessions, five assesstions showed SPFMV infection in PCR based detection. It is understandable from the study that eventhough all plant sample taken for study had clear cut symptoms, they didn't show positive for SPFMV which may be due to interferrence of mucilagenous substances. This needs further modification. The samples which showed positive for SPFMV are SH710, S1294, S275, S940, and S971. The major symptoms commonly exhibited by these samples are feathering, veinal chlorosis, faint to distinct chlorotic spots with or with out purple margins.

Babu *et al.* (2011) carried out RT-PCR assay using potyvirus specific primers (MJ1/MJ2) designed from the core of the coat protein yielding 327 bp amplicon. Amplicons were sequenced and virus specific cDNA probe was generated for Nucleic acid spot hybridization (NASH). The successful biotinylated NASH led to the diagnosis of SPFMV from sweet potato.

In this study molecular characterization of samples were done through RT-PCR using potyvirus specific primers MJ1/MJ2 and SPFMV specific primers SPFMV1 (F) /SPFMV2 (R), amplifying the WCIEN and QMKAA motif of CP and partial CP giving an amplicon of 327 and 411 bp respectively which showed its robustness in detecting SPFMV infections. Degenerate primes to conserved regions in the viral genomes used in identification of potyviruses, they also enable partial genomic sequencing for taxonomic purposes whereas the group specific PCR and subsequent sequence analysis has been usd for rapid detection of potyviruses occurring in mixed infectins (Colinet *et al.*, 1998).

PCR amplified products obtained using sample SH710 were cloned and sequenced to confirm the presence of SPFMV. Out of 8 clones sequenced, The 408 nts SPFMV sequence obtained in the five clones shows maximum similarity of 99% to Argentina isolate SPFMV-O-Arg polyprotein gene (NCBI ID: KF386013.1). The 407 nts SPFMV sequence in two clones shows maximum similarity of 98% with Korean isolate named CW137 (NCBI ID: KP115608.1). The 305 nts sequence

obtained in a single clone shows maximum similarity with Chineese isolate named Shaanix2 (NCBI ID: HQ844123.1). According to Fauquet *et al.* (2005) potyvirus isolate with 85% sequence identity or more over the whole genome are usually considered to be from the same species.

But the further genome sequencing can't be completed, since we only designed the primer based on the whole geonome sequence of Argentina isolate. As a future line of work, primers based on the conserved regions of the three isolates need to be designed.

SUMMARY

6. SUMMARY

A study on "Molecular characterization of *Sweet potato feathery mottle virus*" was conducted at the ICAR-Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram, during 2016- 2017. The important findings of the above studies are summarised in this chapter.

The study conducted using sweet potato germplasm maintained in ICAR-CTCRI showed high symptom severity and increased aggressiveness for potyviruses but SPFMV infection get decreased compared to earlier years. RNeasy plant mini kit protocol or lithium chloride protocol can be used for the isolation of good quality RNA from the sweet potato leaf samples. Due to easily degradable nature of RNA, it was converted into cDNA for PCR analysis and further work.

Among the samples collected from 45 accessions, five are of confirmed with SPFMV infection. RT-PCR based diagnostics carried out using potyvirus specific primer MJ1/MJ2 and SPFMV1 (F)/SPFMV2 (R) revealed the SPFMV infection by giving amplicon of 327 bp and 411 bp respectively.

Out of 45 accessions, five accessions showed SPFMV infection in PCR based detection. It is understandable from the study that eventhough all plant sample taken for study had clear cut symptoms, they didn't show positive for SPFMV which may be due to the interference of mucilagenous substances of sweet potato and this needs to be refined further. The samples which showed positive for SPFMV are S710, S1294, S275, S940, and S971. The major symptoms commonly exhibited by these samples are feathering, veinal chlorosis and distinct-to-faint chlorotic spots having no margins to margins of purple colour.

Cloning and sequencing of amplified products obtained from sample SH710 confirmed is the presence of SPFMV. Out of 8 clones sequenced it was found that clones shows high variation which comprises, Five Argentina isolate named SPFMV-

O-Arg polyporotein gene (NCBI ID: KF386013.1), two Korean isolate named CW137 (NCBI ID: KP115608.1) and one Chinese isolate named Shaanix2 (NCBI ID: HQ844123.1) with 99%, 98% and 91% similarity respectively. It further showed the possibility of presence of RC-strain O-strain and EA-strain in kerala.

By restriction analysis and sequence analysis, variability was detected within SPFMV in Kerala. From the phylogenetic tree constructed, it was observed that the sequences of samples showed high varialbility eventhough lies within the group. Further investigation is necessary to study the extent of variation and to identify the further genomic structrure of the virus.

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94

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APPENDIX

8. APPENDIX

1. CTAB RNA 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 RNA extraction)

Autoclave and store at room temperature

2. Chloroform-Isoamyl alcohol (24: 1)

Chloroform	:	24 ml
Isoamyl alcohol	:	1 ml

3. TAE Buffer (50X)

Tris base	:	242g
Glacial acetic acid	:	57.1 ml
1.5 M EDTA (pH 8.0)	:	100 ml

4. DEPC water

DEPC	:	0.1 ml
Distilled water	:	100 ml

0.1 ml of DEPC in 100 ml of the solution to be treated and shake vigorously

5. Luria Agar Medium

35 g of LA (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

6. Luria Broth Medium

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

7. Preparation of LA Ampicilin/Xgal/IPTG Plates

a. Ampicillin stock (50 mg/ml)

Dissolve 2.5 g Ampicillin in 50 ml deionized water. Filter sterilize and store at -20°C in the dark

b. X gal stock (20 mg/ml)

Dissolve 0.2 g X gal in 10 ml N, N Dimethylformamide. Store at -20°C in the dark

c. IPTG stock (100 mM)

Dissolve 1.7 g IPTG in 50 ml deionized water. Filter sterilize and store at 4°C in the dark.

Before pouring the plates, allow the LA medium (1L) to cool to 55°C, then add 1ml ampicillin stock (making the final concentration to 50µg/ml). Mix gently and pour the plates. Dry the plates opened at room temperature under UV light for 30 min. Add 40µl X gal stock solution to the plate and spread evenly using a sterile spatula. Allow it to diffuse completely into the medium for about 10 min. Then add 40µl IPTG stock solution, spread evenly using a sterile spatula.

Pre-warm the LA ampicillin /X gal /IPTG Plates at 37°C for 20 min before use.

8. Reagents required for plasmid isolation

a. Buffer P1

Tris (pH 8.0)	:	50 mM
EDTA(pH 8.0)	:	10 mM

Autoclave and store at 4°C

b. Buffer P2 (freshly prepared)

NaOH	:	200mM
SDS	:	1 %

Autoclave and store at room temperature

c. Buffer P3

Potassium acetate : 3 M

Adjust the pH to 5.5 with glacial acetic acid. Do not autoclave. Use autoclaved distilled water. Store at 4°C.

104

ABSTRACT

MOLECULAR CHARACTERIZATION OF *SWEET POTATO FEATHERY MOTTLE VIRUS*

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Abstract of the thesis
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9. ABSTRACT

A study on "Molecular characterization of *Sweet potato feathery mottle virus*" was conducted at the ICAR- Central Tuber Crop Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017.

During the study 45 accessions of sweet potato from different fields of ICAR-CTCRI were selected and different symptoms of *Sweet potato feathery mottle virus* infection were recorded. The study reveals that the rate of infection is still prevailing in Kerala having high symptom severity due to combination with other potyviruses and increased aggressiveness as compared to earlier years because of the presence to different strains.

Total RNA was isolated from all samples and used for RT-PCR analysis by degenerate primers for potyviruses and specific primers for SPFMV to differentially identify the presence of SPFMV. From the 45, five were confirmed as SPFMV positive. Among this amplified products obtained from sample SH710 was sequenced. From the 8 clones sequenced 3 different isolates of SPFMV is obtained, *Sweet potato feathery mottle virus* isolate SPFMV-O-Arg polyprotein gene (NCBI ID: KF386013.1) from Argentina, *Sweet potato feathery mottle virus* isolate CW137 (NCBI ID: KP115608.1) from Korean and *Sweet potato feathery mottle virus* isolate Shanxi2 coat protein gene (NCBI ID: HQ844123.1) from China, these are further belongs to broad class of Ordinary and Russet crack and East African. It further showed the possibility of presence of RC-strain O-strain and EA-strain in Kerala.

To identify the phylogenetic relationship of the sequenced samples with that of available accessions, dendrograms were made using MEGA 7.0.26 software and the tree showed that sequences has variability eventhough lies within the group. By restriction analysis and sequence analysis, variability was recorded within SPFMV in Trivandrum.