IDENTIFICATION AND CHARACTERIZATION OF VIRUSES IN SWEET POTATO

[*Ipomoea batatas* (L.) Lam.]

by

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(2010-09-102)

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DECLARATION

I hereby declare that the thesis entitled **"Identification and characterization of viruses in sweet potato [*Ipomoea batatas* (L.) Lam.]"** 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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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
bp	Base pair
cm	Centimetre
m	Meter
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
DNA	Deoxyribo nucleic acid
RNA	Riboxy nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
FP	Forward primer
RP	Reverse primer
FAOSTAT	Food and Agriculture Organization Statistical Database
g	gram
h	Hour
ha	Hectare
kbp	Kilo basepair
kg	Kilogram
M	Molar
mg	milligram
min	Minute
ml	Millilitre
mm	Millimeter
m*M*	Millimolar
NaCl	Sodium chloride
ssRNA	Single stranded RNA

SPFMV	Sweet potato feathery mottle virus
SPMMV	Sweet potato mild mottle virus
SPLCV	Sweet potato leaf curl virus
SPCFV	Sweet potato chlorotic fleck virus
SPCSV	Sweet potato chlorotic stunt virus
SPVG	Sweet potato virus G
SPVC	Sweet potato virus C
SPV2	Sweet potato virus 2
SPVD	Sweet potato virus disease
nm	Nanometer
ORF	Open Reading Frames
polyA	Polyadenelation
mRNA	Messenger RNA
PNG	Papua New Guinea
ELISA	Enzyme Linked Immunosorbent Assay
PCR	Polymerase Chain Reaction
NASH	Nucleic Acid Spot Hybridisation
NCM	Nitrocellulose membrane
DIBA	Dot Immuno Binding Assay
RT	Reverse transcription
CP	Coat protein
BLAST	Basic Local Alignment Search Tool
kDa	Kilo Dalton
RNaseH	Ribonuclease H
AMV-RT	Avian myeloblastosis virus reverse transcriptase
USA	United States of America

INTRODUCTION

1. INTRODUCTION

Ipomoea batatas (L) Lam. is a dicotyledonous perennial plant belonging to the family *Convolvulaceae*. It is commonly known as sweet potato which is the only food crop in the *Convolvulaceae* family. Sweet potato ranks seventh in global food crop production and is the third most important root crop after potato and cassava. It is grown on 8.1 million ha, yielding ca 103 million tones, with an average yield of about 12/ha (FAOSTAT, 2015). It is mainly grown in developing countries which accounts for over 95% of world output. Sweet potato is cultivated on about two lakh hectares of land in India, yielding ca 1 million tones (FAOSTAT, 2015). And 312 ha (2010-11) of land in Kerala are under sweet potato cultivation with production of 4887 tones (FIB, 2013).

Plant viruses are economically important in developing countries that are heavily dependent on agricultural production for food security, employment and export earnings. As sweet potato is a vegitatively propagated plant with vine cuttings, soil borne pathogens are not the causatives for principal diseases causing degeneration over generations. Instead many viruses infect the crop worldwide (Clark *et al.*, 2012).

Sweet potato production is greatly constrained particularly by viral diseases that cause yield reduction of over 50 percentage (Gibson *et al.*, 1998; Mukasa *et al.*, 2006). More than 30 viral diseases of sweet potato have been reported in different parts of the world (Kashif *et al.*, 2012). Sweet potato viruses are mainly spread through healthy looking vines, which farmers collect from the previous crop for the next cropping cycle. Thus singly infected vines can act as source of inoculums and through vector transmissions lead to mixed infections of different viruses (Rukarwa *et al.*, 2010). The most important and devastating viral diseases affecting sweet potato worldwide is Sweet potato virus disease (SPVD) which can reduce yields of infected plants by up to 80 percentage (Mukiibi, 1977; Hahn, 1979). Multiple virus

infection in sweet potato is a common phenomenon (Gibson *et al.*, 1998; Karyeija *et al.*, 2000) and SPVD is caused by synergetic interaction between a *Potyvirus*, *Sweet potato feathery mottle virus* (SPFMV) and a *Crinivirus*, *Sweet potato chlorotic stunt virus* (SPCSV). *Sweet potato feathery mottle virus* (SPFMV) is the widespread virus infecting sweet potato in India. *Sweet potato leaf curl virus* (SPLCV) is another important virus with leaf curl symptoms. Five main viruses detected infecting 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* (SPCFV) and *Sweet potato leaf curl virus* (SPLCV). They have been well studied and characterized when they infect individually. But there is a lack of data on the mixed infections caused by the synergetic interaction of two or more viruses to ensure quality sweet potato planting materials.

Production of virus free planting material is essential for effective management of the viral diseases. To fulfill this requirement the present study was undertaken with an objective to diagnose, clone and characterize viruses implicated in mixed infections of sweet potato.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and tuber crops form the means of sustenance for millions of people in the tropical and sub-tropical world. They have the history of saving mankind in times of famine. They are also an important source of animal feed and industrial products. The world's total harvested area of tubers is nearly 51 million hectares with one third found in Africa and one third in Asia and Pacific regions. On a global basis, approximately 45% of root and tuber crop production is consumed as food, with the remainder used as animal feed or for industrial processing for products such as starch, distilled spirits, and a range of minor products. These crops are recognized as the most efficient converters of solar energy. Apart from that these tubers are known to supply cheap source of energy especially for the weaker sections of the population (Hutabarat and Maeno, 2002).

2.2 SWEET POTATO (*Ipomoea batatas* L.)

Sweet potato (*Ipomoea batatas* L.) is a dicotyledonous perennial plant belonging to the *Convolvulaceae. I. batatas* is the only food crop out of the approximately 500 species in this family (Watson and Dallwitz, 1991; 1994; Onwueme and Charles, 1994). Sweet potato is an important crop for food security (Gibson *et al.*, 2009). It is valued by subsistence farmers because it can produce a crop with few production inputs, withstands stresses such as drought, in the absence of frost it can be left in the field to harvest when needed, and it can also be sold for cash (Karyeija *et al.*, 1998). It is a root, not a tuber, and belongs to the morning-glory family. Many parts of the plant are edible, including leaves, roots, and vines, and varieties exist with a wide range of skin and flesh color, from white to yellow-orange and deep purple. It is grown for green leaves as well as for tubers rich in

carbohydrate and beta-carotene. It is vegetative propagated from vines, tubers or sprouts (root slips). Quality of propagation material makes differences in the yields.

Sweet potato ranks fourth in importance in the developing world after rice, wheat, and corn (Kays, 2005). It is ranked seventh in global food crop production and is the third most important root crop after potato and cassava. In 2013, it is grown on 8.1 million ha, yielding ca 103 million tones, with an average yield of about 12/ha (FAOSTAT, 2015). It is mainly grown in developing countries, which accounts for over 95% of world output. The cultivated area of sweet potato in China is 3.7 million hectares. It accounts for 70% of the total area of sweet potato cultivation in the world. China is the highest producer with production of 80 million tons followed by Vietnam. Yields vary according to the area or locations. The average yield in Africa is about 4.7t/ha, Asia is 20.0t/ha, South America is 12.3t/ha and United States is 22.8t/ha (FAOSTAT, 2012). About 2 lakh hectares of land is under sweet potato cultivation in India yielding ca 1 million tones (FAOSTAT, 2015), cultivated mostly in Odissha, West Bengal, Bihar and Uttar Pradesh. In Kerala, 312 ha (2010-11) of land are under sweet potato cultivation with production of 4887 tones (FIB, 2013).

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

2.2.1 Origin and distribution

Sweet potato is speculated to have originated more than 5000 years ago between Central and northern South America (Huang and Sun, 2000). The crop is now grown in tropical, sub tropical and warm temperate regions between 40°N and 32°S latitude and at elevations up to 2500 m (He *et al.*, 1995). The plant is tolerant to a wide range of soil conditions, but is sensitive to water logging. The crop is generally grown on fairly infertile soils with little inputs of fertilizer. It is believed that after 1492 Portuguese explorers took sweet potato to India, South East Asia, East Indies and Africa (Austin and Daniel, 1988; Zhang *et al.*, 2004; Srisuwan *et al.*, 2006). The vast majority of sweet potato production remains in eastern Asia, according to FAO approximately 80 percent of the global crop being produced in China. Sweet potato is cultivated in more than 100 countries worldwide including Central America, South America, North America, Pacific Islands, India, Africa, Australia, the Caribbean and the Mediterranean basins.

2.2.2 Morphology

The sweet potato is a herbaceous and perennial plant. The types of growth habit of sweet potatoes are erect, semi erect, spreading, and very spreading. Its root system consists of fibrous roots that absorb nutrients and water, and anchor the plant, and storage roots that are lateral roots, which store photosynthetic products. As the plant matures, thick pencil roots that have some lignifications are produced. Stem is cylindrical and its length, like that of the internodes, depends on the growth habit of the cultivar and of the availability of water in the soil. The erect cultivars are approximately 1 m long, while the very spreading ones can reach more than 5 m long. Depending on the sweet potato cultivar, the stem color varies from green to totally pigmented with anthocyanins (red-purple color). Leaves are alternate heart-shaped or palmately lobed. The shape of the general outline of sweetpotato leaves can be rounded, reniform (kidneyshaped), cordate (heart-shaped), triangular, hastate

(trilobular and spear-shaped with the two basal loves divergent), lobed and almost divided. The flower is bisexual. The color of the flower bud pedicel, and peduncle varies from green to completely purple pigmented. The fruit is a 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. The intensity of the color depends on the environmental conditions where the plant is grown (Huaman, 1992). Diagrammatic representation in Figure 1.

2.2.3 Nutritional value

Raw sweet potatoes are rich in complex carbohydrates, dietary fiber and beta-carotene (a provitamin A carotenoid). It also contains other micronutrients, including vitamin C, vitamin B5, vitamin B6, copper, vitamin B1, vitamin B2, phosphorus and manganese. It has moderate levels of iron and zinc. Sweet potato provides less edible energy and protein per unit weight than cereals but it has higher nutrient density. The Center for Science in the Public Interest in 2013 ranked the nutritional value of sweet potatoes as highest among several other foods. A study of 10,000 households in Uganda found that children eating beta-carotene enriched sweet potatoes suffered less vitamin A deficiency than those not consuming as much beta-carotene (Coghlan, 2012). Nutritionists in the USA are exploring the potential cancer preventing properties of purple-fleshed sweet potato. The anthocyanins that account for the purple pigmentation in this variety (also found in fruit and vegetables such as blueberries and red cabbage) are powerful antioxidants and have good bioavailability. The nutrient content per 100mg of raw sweet potato is given in the Table 1.

2.3 VIRAL DISEASES IN SWEET POTATO

Sweet potato production is greatly constrained, particularly by viral diseases that cause yield reduction of over 50% (Gibson *et al.*, 1998; Mukasa *et al.*, 2006). Vegetative propagation should result in the buildup of viruses from generation to generation (Okpul *et al.*, 2011). Sweet potato viruses are mainly spread through healthy looking vines, which farmers collect from the previous crop for the next cropping cycle. Thus singly infected vines can act as source of inoculums and through vector transmissions lead to mixed infections of different viruses (Rukarwa, *et al.*, 2010).

2.3.1 Viruses infecting Sweet potato

More than 30 viral diseases of sweet potato have been reported in different parts of the world (Kashif *et al.*, 2012). The most important and devastating viral disease affecting sweet potatoes worldwide is Sweet potato virus disease (SPVD). Sweet potato virus disease can reduce yields of infected plants by upto 80 per cent (Mukiibi, 1977; Hahn, 1979). Common viral diseases in sweet potato are caused by *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato yellow dwarf virus* (SPYDV) and *Sweet potato leaf curl Georgia virus* (SPLCGV). Multiple virus infections in sweet potato are a common phenomenon (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). SPVD is caused by synergetic interaction between a potyvirus, SPFMV and a crinivirus, SPCSV. SPMMV has occurred most frequently in mixed infections with SPCSV (Mukasa *et al.*, 2003). SPMMV has also occurred in complex with SPCSV and SPFMV (Ateka *et al.*, 2004; Mukasa *et al.*, 2004). The viruses infecting sweet potato are given in Table: 2.

2.3.2 Viruses infecting Sweet potato in India

The common viral diseases seen are SPFMV, SPMMV, *Sweet potato latent virus* (SPLV), *Sweet potato chlorotic fleck virus* (SPCFV) and *Sweet potato leaf curl virus* (SPLCV). *Sweet potato feathery mottle virus* was detected in different samples in India. Electron microscopy studies revealed that the SPFMV is a potyvirus with an average length of 748 nm. The virus was purified from SPFMV infected sweet potato leaves. The antiserum was produced and tested using Ouchterlony agar double-diffusion test. *Sweet potato feathery mottle virus* was detected in different samples using direct antigen coating-ELISA and nitrocellulose membrane-ELISA (Jeeva *et al.*, 2004). Babu *et al.* (2011) carried out reverse transcription polymerase chain reaction 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. Makeshkumar *et al.* (2007) observed some of the sweet potato lines with leaf curl symptoms. Total DNA was isolated from the infected plants and subjected to PCR using gemini group specific primer which has yielded positive amplification of 530 bp. Analysis of the sequence of PCR products showed close relationship with published SPLCV sequences. It is the first report of occurrence of SPLCV in India. Prasanth and Hegde (2008) collected cuttings from symptomatic sweet potato plants from Kerala, Odissha and Andhra Pradesh and maintained in insect-proof glass house. Total nucleic acids isolated from collected sweet potato samples were used for PCR and (RT)-PCR with gemini virus group specific primer and potyvirus specific primer. The expected 530 bp and 1.3 kb fragments were generated from the gemini virus and potyvirus primer sets, respectively. To further identify the viruses, nested primers specific for the coat protein gene of SPFMV and SPLCV were designed. Phylogenetic analysis with MEGA software program showed the highest sequence similarity with SPLCGV.

2.3.3 Potyvirus

The family *Potyviridae* is the largest family of positive-sense, single-stranded RNA (ssRNA) plant viruses currently recognized, many of which cause significant losses in agricultural, pasture, horticultural and ornamental crops. Based on their transmission vectors and genomic characteristics, the members of the family are classified into eight genera, *Potyvirus, Ipomovirus, Macluravirus, Tritimovirus, Bymovirus, Rymovirus, Brambyvirus and Tritimovirus*. Among these the genus *Potyvirus* containing the largest number of plant virus species, including 111 recognized species and 86 tentative species currently assigned to it by the International Committee on Taxonomy of Viruses (ICTV, 2013).

Potyviruses cause significant losses in a wide range of crop plants and are transmitted by aphids in a non-persistent manner. Members of the genus *Potyvirus* have flexuous filament virions, 680-900 nm long and 11-13 nm wide. The single-stranded, positive-sense monopartite or bipartite RNA genome of potyviruses (~ 10^{3} kb) is polyadenylated at the 3' end and has a viral genome-linked protein (VPg) covalently linked to the 5' end.

2.3.3.1 Sweet potato feathery mottle virus (SPFMV)

Sweet potato feathery mottle virus Genus *Potyvirus* (SPFMV) is the most important and widespread virus among the viruses detected in sweet potato (Moyer and Salazar, 1989). SPFMV was first described in the United States about 60 years ago (Tairo *et al.*, 2005). SPFMV is transmitted non-persistently on the stylet tips of aphids as they bite the sweet potato plant (Moyer and Cali, 1985). In Africa, SPFMV causes a SPVD in a complex infection with the whitefly-transmitted Sweet potato sunken vein virus Genus *Crinivirus Sweet potato chlorotic stunt* (SPCSV). Since SPFMV is not as lethal as some, its ability to travel long distances is more damaging when packaged with more virulent viral genomes (Sakai *et al.*, 1997).

Many infections are localized, mild, and often asymptomatic, and can go untreated without causing significant damage to the plant (Karyeija *et al.*, 2000). The most common symptom of SPFMV is a feathery, purple pattern in the leaves (Ryu *et al.*, 1998). The SPFMV genome is approximately 10,820 bases long, varying slightly depending upon the specific strain (Yamasaki *et al.*, 2010). The genome is 10-15% longer than average potyvirus genome lengths; fittingly, cistron also is uniquely large in this virus. The genome consists of single-stranded linear RNA, with a poly(A) region. The virion is a long, flexuous, rod shaped unit, and ranges from 810 to 865 nanometers in length (Abad and Moyer, 1992). SPFMV is transmitted in a non persistent manner by aphids, including *Aphis gossypii*, *Myzus persicae*, *A. craccivora*, and *Lipaphis erysimi*. It is a well-researched target for plant immunity as SPFMV is the most widely spread offender. Genetic modification is one of the predominant methods by which sweet potato plants are protected. Plant cells that undergo transfection with plasmids containing antiviral genes have been observed to successfully develop transgenic plants (Sivparsad and Gubba., 2014).

2.3.3.2 Sweet potato latent virus (SPLV)

Sweet potato latent virus Genus *Potyvirus* (SPLV) is widespread in China and has been reported also from Egypt. Makeshkumar *et al.* (2001) reported SPLV in sweet potato germplasm collection. SPLV may cause mild chlorosis but in most cultivars the infection is symptomless. Symptoms often disappear after infection, but the plants remain infected. SPLV isolates from Japan and China were transmitted by the aphid *Myzus persicae* (Usugi *et al.*, 1991). The virus has flexuous, filamentous particles of approximately 700-750 nm long and induces typical cylindrical inclusion proteins in the cytoplasm of infected cells. The experimental host range of SPLV is wider than that of *Sweet potato feathery virus* (SPFMV). SPLV is serologically related to, but distinct from SPFMV. The best way to control this virus, as well as other viruses infecting sweet potato is by establishing propagation nurseries derived from virus-tested mother plants.

2.3.3.3 Sweet potato mild mottle virus (SPMMV)

Sweet potato mild mottle virus Genus *Ipomovirus* (SPMMV) has been reported from West and South Africa, Indonesia, China, Philippines, India, New Zealand and Egypt. SPMMV can cause leaf mottling, stunting, and loss of yields. The virus is transmitted semi persistently by *Bemisia tabaci*. The virus was transmitted to plants in 14 families (Mcgregor *et al*., 2009). Virions are flexous rod-shaped particles, 800–950 nm in length, containing 5% RNA and 95% protein. The genome consists of single-stranded RNA. The viral RNA was cloned and the assembled genomic sequence was 10,818 nts in length with a polyadenylated tract at the 3-terminus.

2.3.4 Geminivirus

Geminiviruses are plant viruses which have single-stranded circular DNA genomes encoding genes that diverge in both directions from a virion strand origin of replication. It is the largest known family of single stranded DNA viruses. *Geminiviridae* includes *Becurtovirus, Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus*, *Turncurtovirus*. The genome can either be a single component between 2500-3100 nucleotides, or, in the case of some begomoviruses, two similar-sized components each between 2600 and 2800 nucleotides. They have elongated, geminate capsids. The capsids range in size from 18-20 nm in diameter with a length of about 30 nm. Begomoviruses with two component (i.e. bipartite) genomes have these components separated into two different particles both of which must usually be transmitted together to initiate a new infection within a suitable host cell. *Mastrevirus* transmission is via various leafhopper species, *Curtoviruses* and *Topocuvirus* species are transmitted by treehopper species and *Begomoviruses* are transmitted by the whitefly species, *Bemisia tabaci*. These viruses are responsible for a significant amount of crop damage worldwide.

2.3.4.1 Sweet potato leaf curl virus (SPLCV)

Sweet potato leaf curl virus Genus *Begomovirus* (SPLCV) has been reported from the United States, Taiwan, Korea, Argentina, India and Japan. Infected plants show vein clearing, interveinal chlorosis, chlorotic spots, upward leaf curling, leaf narrowing, purpling, blistering and leaf yellowing. The virus is transmitted by *B. tabaci* in a persistent manner (ICTVdB Management, 2006). The virus can be eliminated by thermotherapy-meristem tip culture (El Far and Ashoub, 2009; Arkorful *et al.*, 2015).

2.4 METHODS OF VIRUS DETECTION

Serological methods like enzyme linked immunosorbent assay (ELISA), dot immunobinding assay (DIBA) and nucleic acid based technique like polymerase chain reaction (PCR) are the most common methods of virus detection and identification. In order to improve sweet potato production and to ensure quality sweet potato planting material, effective diagnostic method is a pre-requisite.

2.4.1 Enzyme linked immunosorbent assay (ELISA)

Since 1970s, ELISA have been used widely and successfully for detection of plant viral diseases (Clark and Adams, 1977; Flegg and Clark, 1979). ELISA is a solid phase heterogeneous immunoassay done in microtitre plates made up of polystyrene or polyvinyl chloride. ELISA techniques include NCM-ELISA and DIBA. ELISA fall into two broad categories: direct and indirect procedures where they differ in the way the antigen–antibody complex are detected. ELISA has major limitations such as its low sensitivity during periods of low virus titre. Moreover serological diagnosis of potyviruses is often imprecise, because of frequent serological cross-reactions between species and biological indexing is very cumbersome (Brunt, 1992). A membrane immuno-binding assay also known as nitrocellulose membrane ELISA (NCM-ELISA) has been used with success to detect

several sweet potato viruses (Abad and Moyer 1992; Gutierrez *et al.* 2003; Mukasa *et al.* 2003; Souto *et al.*, 2003; Tairo *et al.*, 2005; Valverde and Moreira, 2004).

2.4.2 Polymerase chain reaction (PCR)

PCR methods for virus detection were first published in the early 1990s (Vunsh *et al.*, 1990) and theoretically offered the user exquisite levels of specificity and sensitivity utilizing gel-electrophoresis for resolution of the results. With the advances in the field of molecular biology, nucleic acid-based methods such as reverse transcription (RT) and the polymerase chain reaction (PCR) began to be used in plant virus detection (Wetzel *et al.*, 1991; Rowhani *et al.*, 1995; Thomson and Dietzgen, 1995; Hsu *et al.*, 2005). Several degenerated primers have been designed to recognize the conserved regions of viral genomes of many virus species or the whole virus genus or family (Langeveld *et al.*, 1991; Bateson and Dale, 1995; Tian *et al.*, 1996; Gibbs and Mackenzie, 1997; Chen *et al.*, 2001; Posthuma *et al.*, 2002).

The available potyvirus sequences in the database made possible the development of a method for the identification of potyviruses based upon the PCR (Langeveld *et al.*, 1991). For PCR based identification of the potyvirus group, local conserved regions in the core domain of the potyvirus coat protein were selected for the construction of degenerate primers for application in a potyvirus group specific combined assay of reverse transcription polymerase chain reaction (RT-PCR) (Babu *et al.*, 2012). The vast majority of degenerate primers have been designed to sequences at the 3'end of the genome, such as the CP- and NIb- coding regions. The use of degenerate primers has not only facilitated the rapid detection of many potyviruses but has also enabled partial genomic sequencing for taxonomic purposes (Ha *et al.*, 2008). 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; Grisoni *et al.*, 2006; Babu *et al.*, 2012). The

comparative sequence analyses study of SPMMV with other potyviruses revealed that NIb is the most conserved protein among members of the family *Potyviridae* (Colinet *et al.*, 1998). The group-specific PCR and subsequent sequence analysis of the amplified regions has been used for rapid detection and identification of Potyvirus and is appeared to be the most suitable method for identification of viruses which are difficult to purify and/or occurring in mixed infections (Colinet *et al.*, 1998).

Due to the characteristic poly (A) tail at the 3' end of potyvirus genome, the first-strand cDNA of potyviruses was synthesized using oligo(dT) 12-18 primer or random hexamers with RNA as the template (Li *et al.*, 1998; Wen-Chi Hu *et al.*, 2010). Hsu *et al.* (2005) developed an RT-PCR based method, which has the potential to detect members of the genus *Potyvirus* by using new designed potyvirus degenerate primers. Combining the RT-PCR technique and degenerate primers, it is possible to detect many virus species of the same genus or family in a single test, but it cannot distinguish the virus species. Currently, rapid detection and identification of a plant virus is based on ELISA, RT-PCR with specific primers, or cloning and sequencing methods. These methods are facilitated when some information about the target viruses is available.

Gemini viruses are well suited to detection and identification by PCR because they replicate via a double-stranded, circular DNA intermediate-the replicative form-which can serve as a template for PCR amplification (Stanley, 1991). Rojas *et al.* (1993) designed degenerate primers coding conserved regions in DNA-A and DNA-B which served as general primers for amplifying fragments of Gemini viruses. Wyatt and Brown (1996) used AV494/AC1048 degenerate primer pair as the universal subgroup III geminivirus specific primers targeting the middle or core region of the coat protein. The primer SPG1/SPG2 has been used to identify several isolates of SPLCV in sweet potato plants due to its high sensitivity as a result of its highly conserved annealing regions of open reading frames AC2 and AC1 (Lotrakul

et al., 1998). Li *et al.* (2004) used the same primers to detect geminivirus in sweet potato.

2.5 CHARACTERISATION OF VIRUSES INFECTING SWEET POTATO

2.5.1 *Sweet potato feathery mottle virus* (SPFMV)

SPFMV is the most thoroughly characterized sweet potato virus (Campbell *et al.*, 1974; Moyer, 1986; Moyer and Kennedy, 1978). The SPFMV genome is approximately 10,820 bases long, varying slightly depending upon the specific strain (Yamasaki *et al.*, 2010). The majority of the SPFMV genome is one open reading frame, followed by a 3' UTR and a poly(A) tail. The 3' UTR exhibits secondary structure that may be involved in recognizing viral replicase. All potyviruses have 3' poly(A) sequences, although they lack the cellular signal sequence for poly(A) tail addition. The encoded genes are P1, HCPro (helper component proteinase), P3, 6K1, CI, 6K2, NIa, NIb, and the coat protein cistron, which is found in a variety of other viruses. During replication, the entire genome is translated as a polyprotein and cleaved.

2.5.2 *Sweet potato latent virus* (SPLV)

Sweet potato latent virus (SPLV), formerly designated as sweet potato virus N, was first reported from Taiwan. Virus particles are flexuous rods, 750–790 nm in length. The capsid protein has a MW of 36,000. The use of potyvirus-specific primers and subsequent application of the RACE procedure allowed the cloning of the 3' terminal 1088 nucleotides of the genomic RNA of the Taiwan isolate of sweet potato latent virus (SPLV-T) and the 3' genomic 1085 nucleotides of a SPLV-like virus from China (SPLV-CH). The sequence of an internal part of the presumptive nuclear inclusion b gene was also determined for both isolates. Presence of consensus motifs indicated that SPLV-CH and SPLV-T should be regarded as

members of the genus Potyvirus. Multiple sequence alignments and phylogenetic analyses revealed SPLV was not related to other potyviruses infecting sweet potato or to any other sequenced virus (Colinet *et al.*, 1997). Nishiguchi *et al.* (2001) found out SPLV has 58% homology to SPFMV-S.

2.5.3 *Sweet potato mild mottle virus* (SPMMV)

The genome consists of single-stranded RNA. The viral RNA was cloned and the assembled genomic sequence was 10,818 nucleotides in length with a polyadenylated tract at the 3-terminus. Almost all known potyvirus motifs are present in the polyprotein of SPMMV, except some motifs in the putative helper-component and CP, which are incomplete or missing. This may account for its vector relations (Colinet, *et al.*, 1998). The CP has a MW of 37,700. A synergism was observed in sweet potato doubly infected by SPMMV and SPCSV (but not by SPFMV) (Untiveros *et al.*, 2008).

2.5.4 *Sweet potato leaf curl virus* (SPLCV)

The monopartite DNA genome is 2,828 nucleotides in length but varied according to the isolates from different locations (Banks *et al.*, 1999; Lotrakul and Valverde, 1999; Luan *et al.*, 2006; and Pardina *et al.*, 2012). Luan *et al.* (2006) showed the PCR expected DNA fragments of 2.8 kb of China isolates contained the AV1, AV2, AC1, AC2, AC3, and AC4 open reading frames. The AC4 showed 92% identity with the nucleotide sequence of corresponding regions of published sequences of SPLCV available in GenBank.

Table 1: Proximate Composition of the Sweet potato on a fresh weight basis

Component	Content (value per 100mg)
Water	86.81g
Energy	42 kcal
Protein	2.49g
Total lipid (fat)	0.51g
Carbohydrate	8.82g
Dietary fiber	5.3g
Calcium	78mg
Iron	0.97mg
Magnesium	70mg
Phosphorus	81mg
Potassium	508mg
Sodium	6mg
Vitamin A	189µg
Vitamin C	11mg
Thiamine	0.156mg
Riboflavin	0.345mg
Niacin	1.130mg
Cholesterol	0mg

Source: United States Department of Agriculture, National Nutrient Database for Standard Reference Release 27.

Table 2: Viruses reported to infect sweet potato

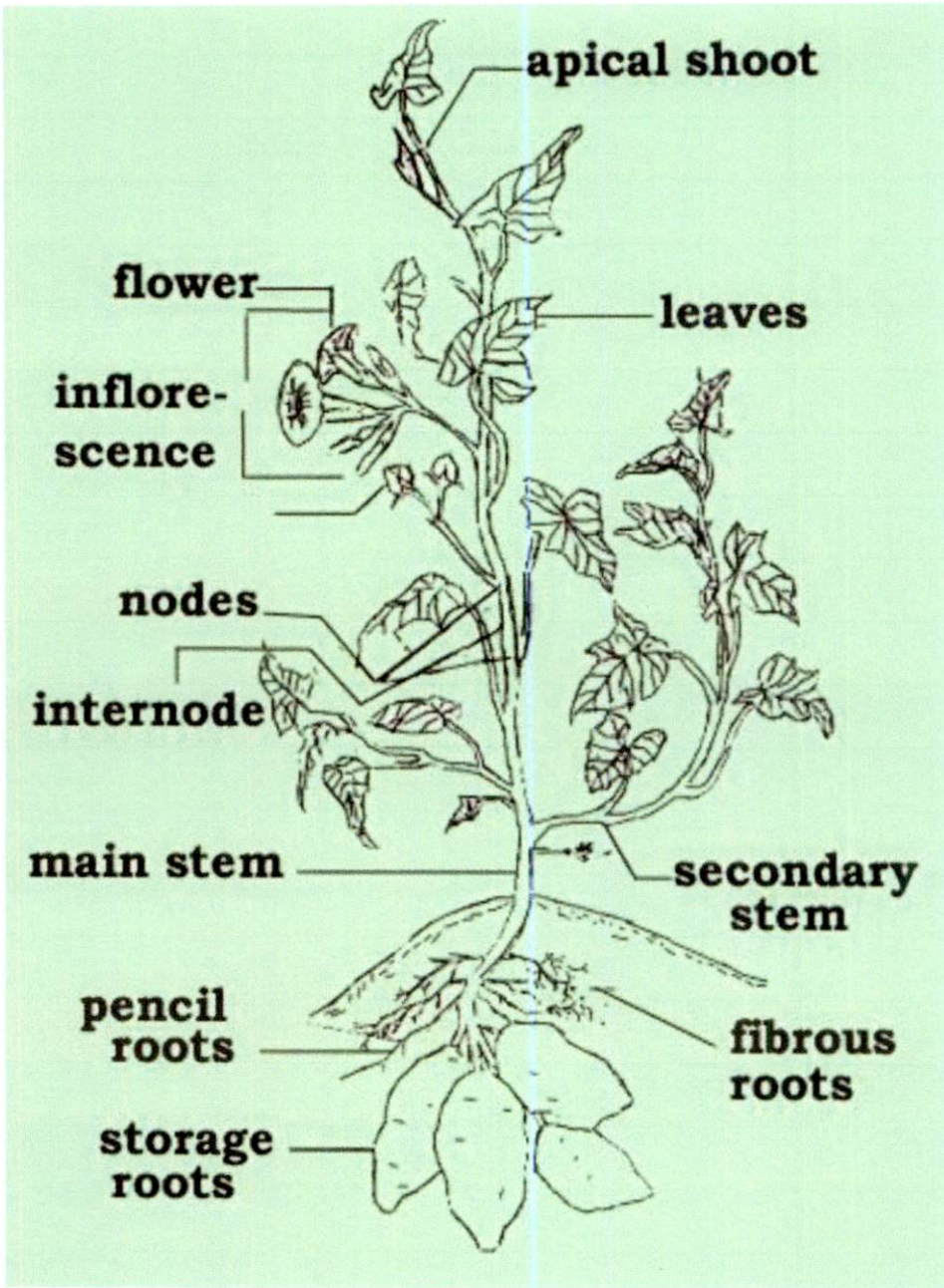
Virus	Family/Genus	Vector	Distribution	Reference
Cucumber mosaic virus (CMV)	*Bromoviridae/Cucumovirus*	Aphids	World-wide, especially in temperate regions.	Cohen *et al.*, 1988
Ipomoea yellow vein virus (IYVV)	*Geminiviridae/Begomovirus*	Whiteflies	Spain and Italy	Lotrakul *et al.*, 2003
Sweet potato chlorotic stunt virus (SPCSV)	*Closteroviridae/Crinivirus*	Whiteflies	Tropics worldwide, sub-Saharan Africa and South America	Winter *et al.* 1992; Kreuze *et al.*, 2002
Sweet potato feathery mottle virus (SPFMV)	*Potyviridae/Potyvirus*	Aphids	World-wide	Moyer and Kennedy, 1978; Sakai *et al.*, 1997
Sweet potato latent virus (SPLV)	*Potyviridae/Potyvirus*	Aphids	Asia [China Taiwan, Japan, Indonesia, Philippines, India], Africa [Kenya and Uganda], Egypt and Peru	Colinet *et al.*, 1997
Sweet potato virus G (SPVG)	*Potyviridae/Potyvirus*	Aphids	China, USA, Taiwan, Japan, Spain, South America, Egypt, Africa	Colinet *et al.*, 1994
Sweet potato leaf curl virus (SPLCV)	*Geminiviridae/Begomovirus*	Whiteflies	Taiwan, Sub-Saharan Africa, USA, China, *India,* Japan, Korea, Brazil, Peru, Israel and Spain	Lotrakul and Valverde, 1999
Sweet potato leaf curl Georgia virus (SPLCGV)	*Geminiviridae/Begomovirus*	Whiteflies	USA, India	Lotrakul *et al.*, 2003
Sweet potato leaf speckling virus (SPLSV)	*Luteoviridae/Enamovirus*	Aphids	CIP's germaplasm collection, Cuba	Fuentes *et al.*, 1996
Sweet potato mild mottle virus (SPMMV)	*Potyviridae/Ipomovirus*	Whiteflies	West Africa, South Africa, Indonesia, China, Philippines, Papua New Guinea, India	Colinet *et al.*, 1996

Table 2 (continued): Viruses reported to infect sweet potato

Virus	Family/Genus	Vector	Distribution	Reference
Sweet potato mild speckling virus (SPMSV)	*Potyviridae/Potyvirus*	Aphids	South America, South Africa, China, Indonesia, Philippines, Egypt, Nigeria and New Zealand	Alvarez *et al.*, 1997
Tomato spotted wilt virus. (TSWV)	*Bunyaviridae/Tospovirus*	Thrips		Clark and Hoy., 2007
Sweet potato chlorotic fleck virus (SPCFV)	*Flexiviridae/Carlavirus*	Mechanical transmission	CIP's germplasm collection, South America, South Asia, South east Asia, Uganda, Australia and New Zealand	Fuentes and Salazar, 1992; Aritua and Adipala, 2004
Ipomoea crinkle leaf curl virus (ICLCV)	*Geminiviridae/Begomovirus*	Whiteflies	Israel, North America	Cohen *et al.*, 1997
Sweet potato ringspot virus	*Comoviridae/Nepovirus*	Mechanical transmission	Papua New Guinea	Brunt *et al.*, 1996
Sweet potato vein mosaic virus	*Potyviridae*	Aphids	Argentina	Nome, 1973
Sweet potato virus 2 (SPV2)	*Potyviridae/Potyvirus*	Aphids	Taiwan, China, Portugal, USA, South Africa, Peru, Australia and Spain	Rossel and Thottaplilly, 1988
Sweet potato yellow dwarf virus (SPYDV)	*Potyviridae/Ipomovirus*	Whiteflies	Taiwan, Far East and Brazil	Liao *et al.*, 1979
Sweet potato pakakuy virus (SPPV) (synonyms Sweet potato badnavirus A and B)	*Caulimoviridae/Badnavirus*	Unknown	Peru, Tanzania	Kreuze *et al.*, 2009; Mbanzibwa *et al.*, 2011
Sweet potato C-3 virus	*Bunyaviridae/Phlebovirus*	Aphids	Brazil	Fuentes and Salazar, 1989: Loebenstein *et al.*, 2009
Sweet potato C-6 virus	*Flexiviridae/Carlavirus*	Mechanical transmission	Cuba, Peru, Philippines, Indonesia, USA,	Loebenstein *et al.*, 2009

Table 2 (continued): Viruses reported to infect sweet potato

Virus	Family/Genus	Vector	Distribution	Reference
Sweet potato collusive Virus(SPCV)(synonym Sweet potato caulimo-like virus)	*Caulimoviridae/Cavemovirus*	Unknown	China, Philippines, Madeira, New Zealand, Papua New Guinea, Australia, Uganda, Kenya, Nigeria, Egypt and Puerto Rico	Cuellar *et al.*, 2011; De Souza and Cuellar, 2011
Sweet potato Golden vein associated virus (SPGVaV)	*Geminiviridae/Begomovirus*	Unknown	Brazil	Paprotka *et al.*, 2010
Sweet potato leaf curl Canary virus (SPLCCaV)	*Geminiviridae/Begomovirus*	Whiteflies	Spain	Lozano *et al.*, 2009
Sweet potato leaf curl China virus (SPLCV-CN)	*Geminiviridae/Begomovirus*	Whiteflies	China	Luan *et al.*, 2007
Sweet potato leaf curl Lanzarote virus (SPLCLaV)	*Geminiviridae/Begomovirus*	Whiteflies	Spain	Lozano *et al.*, 2009
Sweet potato leaf curl Spain virus (SPLCESV)	*Geminiviridae/Begomovirus*	Whiteflies	Spain	Lozano *et al.*, 2009
Sweet potato leaf curl South Carolina virus (SPLCSCV)	*Geminiviridae/Begomovirus*	Whiteflies	USA	Ling *et al.*, 2010; Zhang and Ling, 2011
Sweet potato leaf curl Uganda virus (SPLCUV)	*Geminiviridae/Begomovirus*	Whiteflies	Uganda	Wasswa *et al.*, 2011
Sweet potato mosaic associated virus (SPMaV)	*Geminiviridae/Begomovirus*	Whiteflies	Brazil, South Africa	Paprotka *et al.*, 2010
Sweet potato symptomless virus 1(SPSMV-1)	*Geminiviridae /Mastrevirus*	Unknown	Peru, Tanzania	Kreuze *et al.*, 2009; Mbanzibwa *et al.*, 2011
Sweet potato vein clearing virus (SPVCV)	*Caulimoviridae/Solendovirus*	Unknown	China	Liao *et al.*, 1979



Huaman, 1992

Figure 1: Sweet potato morphology

Figure 2: Symptoms of Sweet potato feathery mottle virus

Figure 3: Symptoms of Sweet potato mild mottle virus

Figure 4: Symptoms of Sweet potato leaf curl virus

Figure 2, 3 & 4 Source: http://keys.lucidcentral.org

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Identification and characterization of viruses in sweet potato (*Ipomoea batatas* (L.) Lam.)" was carried out at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2014-2015. 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 CTCRI, Thiruvananthapuram and Regional Centre-CTCRI, Bhubaneswar. 37 leaf samples exhibiting viral symptoms (i.e. plants showing characteristic irregular chlorotic patterns (feathering), veinal chlorosis, faint-to-distinct chlorotic spots with or without purple margins, stunting, puckering, upward curling or rolling of leaves) and suspected of virus infection were collected. From these, 32 samples with various symptoms were selected as the representative sample set for the present study after initial serological screening. Plants showing no observable symptoms were also sampled to check the possibility of latent infection. The samples were photographed, symptoms were recorded and stored. The geographical origin of selected accessions is represented in Table 5.

3.2 SEROLOGICAL METHODS OF DETECTION

Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immunobinding Assay (DIBA) were employed for the detection of virus infection in *Ipomoea batatas* leaf samples. This procedure was employed to screen the samples for SPFMV and SPMMV infection using SPFMV, SPMMV polyclonal antibodies obtained from DSMZ, Germany.

3.2.1 Enzyme Linked Immunosorbent Assay (ELISA)

Plate Trapped Antigen ELISA (PTA-ELISA) for detecting potyviruses-SPFMV and SPMMV were carried out on 32 *I. batatas* leaf samples showing different symptoms of virus infection. Respective positive samples gifted by DSMZ were used as positive controls and a healthy non host sample collected from the field was used as the negative control.

Two hundred microlitre of the samples extracted in coating buffer were added to the wells of microtitre plates and incubated at 37°C for 2-4 hrs. Following the incubation, the plates were washed with PBS-T buffer thrice for 3 min each. 200 µl of blocking solution (2% skim milk dried powder in 1X PBS-T) was added to each well and incubated for 30 min at 37°C. The blocking solution was removed and the plate was tapped dry. The wells were then incubated with potyvirus specific antisera diluted in PBS-T (1:1000 v/v) for 2 h at 37°C. After discarding the antiserum and washing thrice in PBS-T, alkaline phosphatase conjugated rabbit antimouse antibody (RAM-AP) (secondary conjugate antibody) diluted to 1:500 v/v in conjugate buffer (PBS-T-PVP + 0.2% BSA) was added to the wells and incubated for 2h at 37°C. The incubation was followed by washing the plate thrice in 1X PBS-T. 200 µl of freshly prepared p-Nitrophenyl Phosphate (pNPP) in substrate buffer (1mg ml^{-1}) was added and incubated for 1-2 hrs. The absorbance value was taken at 405 nm (A_{405}) using ELISA reader (Bio-Rad).

3.2.2 Dot-Immunobinding Assay (DIBA)

Thirty three representative leaf samples and a healthy non host were screened for DIBA using SPFMV and SPMMV antibody. Desired sizes of NCM were cut and 1cm^2 squares were drawn on it. The NCM wetted by floating it in TBS and were air dried. 5 µl of partially purified *Ipomoea batatas* leaf samples were spotted on respective squares. After air drying, the membranes were immersed in blocking

solution (Appendix) with gentle shaking for 1 h at room temperature. They were then rinsed once in TBS for 10 min. This was followed by incubating the membranes with primary antibodies respective to the SPFMV and SPMMV (Polyclonal SPFMV IgG and SPMMV IgG) diluted to 1:1000 in TBS-SDM for 1 h at room temperature or overnight at 4°C. Then the membranes were washed thrice with TBS at 10 min interval and incubated with secondary antibody (enzyme labelled anti-rabbit IgG (ALP-conjugate) diluted 1:500 in TBS-SDM for 1 h at room temperature or overnight at 4°C. After rinsing thrice with TBS, the NCM were incubated in substrate solution (BCIP/NBT) at room temperature in dark condition for 10-15 min. They were then observed for color development. The membranes were rinsed with distilled water and then air dried. The color formation and intensity analyzed.

3.3 NUCLIEC ACID EXTRACTION

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

3.3.1 Standardization of DNA isolation protocol

The extraction of good quality DNA from sweet potato was difficult owing to the presence of high amounts of polyphenol and mucilage.

3.3.1.1 DNA Isolation

For genomic DNA isolation, some modifications were done on CTAB method of DNA extraction (Doyle and Doyle, 1990). β-mercaptoethanol was added fresh to the CTAB extraction buffer (Appendix) to give a final concentration of 2% (v/v). The buffer was pre-heated to 60°C in water bath (ROTEK, India). The samples (100 mg) were chilled and pulverized to fine powder by 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 and then 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 room temperature. The supernatant was transferred to another sterile microfuge 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 and centrifuged (Hermle, Table top refrigerated centrifuge) at 15000 rpm for 10 min at room temperature. 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 15000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was washed in 0.5 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).

3.3.2 Analysis of the extracted DNA

3.3.2.1 Agarose gel electrophoresis

The integrity and quality of the extracted DNA was checked in agarose gel electrophoresis. An agarose gel of 1 percent was prepared in 1X TAE buffer (Appendix) and 0.5 µl per litre ethidium bromide was added. Five microlitre of DNA sample with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 Vcm^{-1} for 30-40 min. The gel was then visualized under UV light and the image was documented using Alpha Imager gel documentation system.

3.3.2.2 Quantification of DNA

The DNA yield and purity was determined by spectrophotometric method. Absorbance readings are performed at 260 nm where the DNA absorbs UV light most strongly. DNA concentration (µg/ml) was estimated by measuring the absorbance at 260 nm, multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 equals 50 µg/ml pure dsDNA. To evaluate DNA purity, the ratio of the absorbance at 260 nm divided by the reading at 280 nm was calculated.

3.3.3 Isolation of total RNA

Leaf samples of virus infected sweet potato plants showing symptoms were taken for RNA isolation. RNA was isolated using Lithium Chloride method. The RNA pellet was solubilized in 50 µl of DEPC treated water and stored at -20°C.

3.3.3.1 RNA isolation using Lithium Chloride Method

Around 100 mg of leaf sample 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) was added, vortexed and incubated at 65°C (Lab ROTEK, India) for 10 min. The tube was centrifuged at 15,000 rpm (Hermle, 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) 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 in at 4°C, the pellet was washed with

75 percent ethanol by centrifuging at 10,000 x g at 4°C. The washing was repeated. The RNA pellet obtained was air dried at 37°C for 30 min and then dissolved in 50 µl DEPC water. After incubating at 37°C for 1 h while tapping intermittently, the RNA was stored at -20°C (Vest frost Low Temperature Cabinet, India).

3.3.4 Analysis of the extracted DNA

3.3.4.1 *Agarose gel electrophoresis*

The integrity and quality of the extracted RNA was checked in agarose gel electrophoresis. An agarose gel of 1.2 percent was prepared in 1X TAE buffer and 0.5 µl per litre ethidium bromide was added. Three microlitre of RNA sample with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 Vcm^{-1} for 30 min. The gel was then visualized under UV light and the image was documented using Alpha Imager gel documentation system.

3.3.4.2 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 at 260 nm, multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 equals 40µg/ml pure RNA. To evaluate RNA purity, the ratio of the absorbance at 260nm divided by the reading at 280 nm was calculated.

3.3.5 cDNA synthesis

3.3.5.1 First strand cDNA synthesis

The RNA isolated from leaf samples were subjected to cDNA conversion using AMV-Reverse Transcriptase (GeNei, Bangalore). The components of the reaction mix were as follows:

RNA	: 5µl
10pmol Oligo-dT	: 1 µl
Nuclease free Water	: 9 µl
10X buffer of AMV	: 2 µl
50 mM $MgCl_2$	: 0.5 µl
200 mM dNTP mix	: 2 µl
AMV RT (5U/ µl)	: 0.5 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 50°C for 1 h followed by an extension step at 85°C for 5 min.

3.4 MOLECULAR DETECTION OF VIRUSES

3.4.1 Polymerase chain reaction based detection

Polymerase chain reaction (PCR) analysis was carried out with the isolated DNA and RNA using genus specific primers (potyvirus) and virus specific primers (SPFMV, SPMMV, SPLCV, SPCFV, SPVG, SPVC, SPV2). The primers (Table 3 and Table 4) were synthesized from Eurofins (India). The synthesized primers (100µM) were diluted to a final concentration of 5 µM with sterile water to obtain the working solution.

3.4.1.2 Analysis of amplicon by agarose gel electrophoresis

The most common method to analyse the PCR product is to run an aliquot of the sample on agarose gel. Aliquot of PCR mix (10 µl) was loaded on agarose gel (1 %) made of 1X TAE buffer. 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.

3.4.2 PCR analysis with potyvirus specific primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10X Taq buffer A	:	2.5 µl
200µM dNTP	:	1 µl
10pmol Forward primer	:	1 µl
10pmol Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase (5000U)	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C, annealing at 51.2 °C for AtropaNad2.1a/AtropaNad2.2b, 55.1°C for NIb2F/NIb3R, 38.8°C for CN48/Oligo-dT, 50°C for MJ1/MJ2, 50°C for MJ1/M4T, 50°C for Pot 1/Hrp-5, 54.8°C for Pot1/Pot2, 50.0°C for Hrp-5/Oligo-dT 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 amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.3 PCR analysis with SPFMV1/SPFMV2 primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10X Taq buffer A	:	2.5 µl
200µM dNTP	:	1 µl
10pmol Forward primer	:	1 µl
10pmol Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase (5000U)	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 93°C 30 sec, annealing at 55 °C 1 min and extension at 72°C for 90 sec. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.4 PCR analysis with MMV1/MMV2 primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10X Taq buffer A	:	2.5 µl
200µM dNTP	:	1 µl
10pmol Forward primer	:	1 µl
10pmol Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase (5000U)	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 96°C for 2 minutes followed by 35 cycles of denaturation at 96°C 30 sec, annealing at 50.6 °C 1 min and extension at 72°C for 90 sec. Final extension was done at 72°C for 8 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.5 PCR analysis with LCV1/LCV2 primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl

10X Taq buffer A : 2.5 µl

200µM dNTP : 1 µl

10pmol Forward primer : 1 µl

10pmol Reverse primer : 1 µl

Template DNA (cDNA) : 2 µl

Taq polymerase (5000U) : 0.3 µl

Total volume : 20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C 1 min, annealing at 55 °C 2 min and extension at 72°C for 3 min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.6 PCR analysis with SPG-F1/SPFCG2-R2 primers

The components of the mixture were optimized as listed below:

Water : 12.2 µl

10X Taq buffer A : 2.5 µl

200µM dNTP : 1 µl

10pmol Forward primer : 1 µl

10pmol Reverse primer : 1 µl

Template DNA (cDNA) : 2 µl

Taq polymerase (5000U) : 0.3 µl

Total volume : 20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C 30 sec, annealing at 55 °C 30 sec and extension at 68°C for 1min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.7 PCR analysis with SPC-F1/SPFCG2-R2 primers

The components of the mixture were optimized as listed below:

Water : 12.2 µl

10X Taq buffer A : 2.5 µl

200µM dNTP : 1 µl

10pmol Forward primer : 1 µl

10pmol Reverse primer : 1 µl

Template DNA (cDNA) : 2 µl

Taq polymerase (5000U) : 0.3 µl

Total volume : 20 µl

PCR was carried out in Eppendorf Mastercycle (Germany). PCR programme was set with initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C 30 sec, annealing at 55 °C 30 sec and extension at 68°C for 1min. Final extension was done at 72°C for 5 min. Control reactions were carried out to distinguish the target products from non target products and primer dimer. The amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.8 PCR analysis with PMB-136/PMB-14 primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10X Taq buffer A	:	2.5 µl
200µM dNTP	:	1 µl
10pmol Forward primer	:	1 µl
10pmol Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase (5000U)	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C 30 sec, annealing at 55 °C 30 sec and extension at 72°C for 60sec. 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

amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.4.9 PCR analysis with PMB-25a/PMB-26 primers

The components of the mixture were optimized as listed below:

Water	:	12.2 µl
10X Taq buffer A	:	2.5 µl
200µM dNTP	:	1 µl
10pmol Forward primer	:	1 µl
10pmol Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase (5000U)	:	0.3 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Master cycle (Germany). PCR programme was set with initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C 30 sec, annealing at 55 °C 30 sec and extension at 72°C for 60sec. 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 amplified products along with PCR Marker (low range) from Genei, Bangalore 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.

3.5 CHARACTERISATION OF VIRUSES

3.5.1 Gel elution of PCR amplified fragments

Extraction of PCR products was carried out with QIAEX II 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 was placed into a 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 II suspension and the tube was incubated at 50°C for 10 min occasionally inverting it every 2 min to solubilise the gel. The sample was centrifuged at 13000 rpm, 15-25°C for 30 s and the flow through was discarded. The pellet was washed with QX1 buffer (500 µl), vortexed, centrifuged for 30 s at 13000 rpm, 15-25°C and the flow through was discarded. Again the pellet was washed twice with PE buffer (500 µl), vortexed, centrifuged for 30 s and the flow through was discarded. The sample tube was air dried for 30 min until the pellet became white. TE buffer (20 µl) was added to the tube, vortexed and incubated at room temperature for 5 min. Then it was centrifuged for 30 s at 13000 rpm, 15-25°C and 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 analyzed using agarose gel (1%).

3.5.2 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) where used for the transformation procedure. The ligation mix was prepared with the components as listed below.

Vector pTZ57R/T	:	3 µl
5 X ligation buffer	:	6 µl
PCR product	:	4 µl
Nuclease free water	:	16 µl
T4 DNA ligase	:	1 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 (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 resuspend 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 (Appendix) 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 reaction 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). The plates were incubated overnight at 37°C. Untransformed DH5α cells were plated on an LB Ampicillin X gal/IPTG agar plate to serve as negative control.

Day 3:

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

3.5.2.1 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:

Water nuclaease free	:	14.2 µl
10X Taq buffer A	:	2.5 µl
200µM dNTP	:	1 µl
10pmol Forward primer	:	1 µl
10pmol Reverse primer	:	1 µl
Template DNA (cDNA)	:	2 µl
Taq polymerase (5000U)	:	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) inorder to save the clone for repropagation. The PCR was performed at the required PCR condition of each primer. The products of colony PCR were resolved on a agarose gel (1%).

3.5.3 DNA sequencing

Gel elutes of PCR products were sequenced at the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Center for Biotechnology (RGCB). Nucleotide BLAST of the obtained sequence was performed inorder to find out the similar sequences.

3.5.3.1 Sequence analysis

The electropherogram obtained by the capillary sequencing was first edited with BioEdit Sequence Alignment Editor program version 7.2.5. The edited sequence was compared to known viral sequences using NCBI BLAST. Phylogenetic tree was constructed from BioEdit aligned sequences using NCBI BLAST analysis tool.

Table 3: List of potyvirus specific primers used for virus screening of the samples

Primer	Annealing Temperature	Product size (bp)	Target Region	Primer Sequence
AtropaNad2.1a/AtropaNad2.2b	51.2	188, 800	NAD	5'-GGACTCCTGACGTATACGAAGGATC-3'/ 5'-AGCAATGAGATTCCCCAATATCAT-3'
Nlb2F/ Nlb3R	55.1	350	NIb region	5'-GTITGYGTIGAYGAYTTYAAYAA-3'/ 5'-TCIACIACIGTIGAIGGYTGNCC-3'
CN48/ Oligo-dT	38.8	700	WCIEN motif of CP	5'-TCGTGIATHGANAATGG-3'/ 5'-$(T)_{21}$V-3'
Pot1/ Pot 2	54.8	1300	NIb and CP	5'- GACTGGATCCATTBTCDATRCACCA-3'/ 5'- GACGAATTCTGYGAYGCBGATGGYTC-3'
Hrp-5/ Oligo-dT	50.0	1500	3'end	5'-ATGATHGARKCNTGGGG-3'/ 5'-$(T)_{21}$V-3'

Table 4: List of virus specific primers used for virus screening of the samples

Primer	Virus	Amplified region	Amplicon size	Primer sequence
SPFMV1/SPFMV2	Sweet potato feathery mottle virus	Partial CP	411 bp	5'-ATAGTGGCATCATCAAAGG-3'/ 5'-CCTAAAAGTAGGCACTGCATG-3'
MMV1/MMV2	Sweet potato mild mottle virus	Partial CP	211 bp	5'-GAATATGGAAGATCAGGAGGTG-3' 5'-AAAGTCAATACCCAACCAAGA-3'
LCV1/LCV2 (SPCP-F/SPCP-R)(Merlin)	Sweet potato leaf curl virus	Partial CP	446 bp	5'-GGATCCAGTACAAGTGGGATT-3' 5'-TTAAAGCTTTTAGTATCAGGA-3'
PMB-136/PMB-14	Sweet potato chlorotic fleck virus	5'end of CP	715 bp	5'- GAAGAGTAGCTCTGAGGTG-3' 5'- CAGGTGCAAAAAGGCGACAGAC-3'
SPG-F1/SPFCG2-R2	Sweet potato virus G	Partial CP	1191 bp	5'- GTATGAAGACTCTCTGACAAATTTTG-3' 5'-TCGGGACTGAARGAYACGAATTTAA-3'
SPC-F1/SPFCG2-R2	Sweet potato virus C	Partial CP	836 bp	5'-GTGAGAAAYCTATGCGCTCTGTT-3' 5'-TCGGGACTGAARGAYACGAATTTAA-3'
PMB-25a/PMB-26	Sweet potato virus 2	5'end of CP	698 bp	5'- CCATATATTGCGGAAACAGC-3' 5'- TCAGATACACCAAACCATGAG-3'

RESULTS

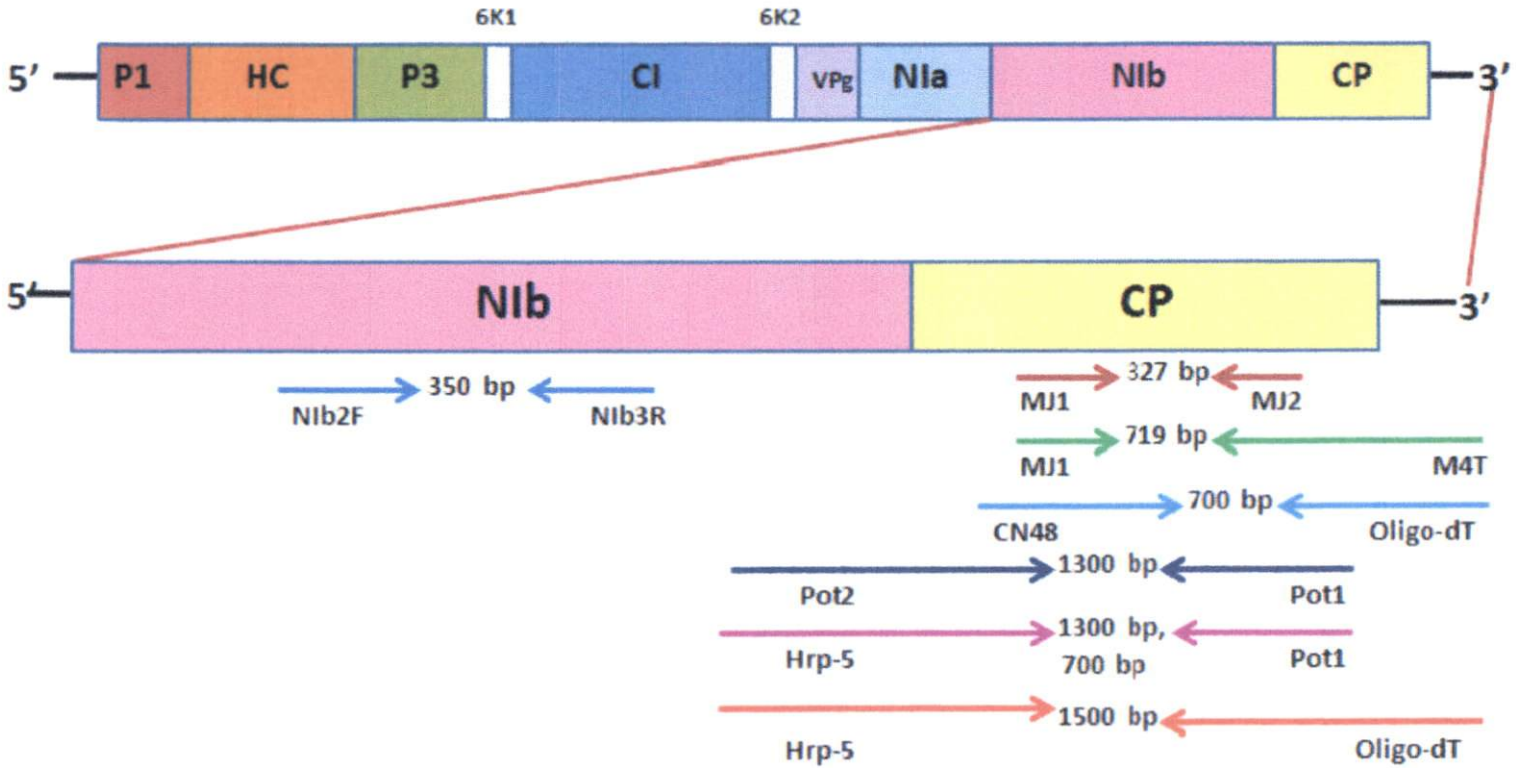


Figure 5: Primers for amplifying various regions of potyvirus genome

4. RESULTS

The results of the study entitled "Identification and characterization of viruses in sweet potato (*Ipomoea batatas* (L.) Lam.)" carried out at the Division of Crop Protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2014-2015 are presented in this chapter.

4.1 SAMPLE COLLECTION

Sweet potato samples belonging to various accessions were collected randomly from the germplasm collection maintained at CTCRI, Thiruvananthapuram and Regional Centre-CTCRI, Bhubaneswar. 37 leaf samples exhibiting viral symptoms were collected. From these, 32 samples with various symptoms were selected as the representative sample set (Plate1) for the present study after initial serological screening. The sample details and symptoms observed are represented in Table 5. This representative sample set was used for further tests and analysis.

4.2 SEROLOGICAL METHODS OF DETECTION

Enzyme Linked Immunosorbent Assay (ELISA) and Dot Immunobinding Assay (DIBA) were employed for the detection of virus infection in *Ipomoea batatas* leaf samples. This procedure was employed to screen the samples for SPFMV and SPMMV infection using SPFMV, SPMMV polyclonal antibodies obtained from DSMZ, Germany.

4.2.1 Enzyme Linked Immunosorbent Assay (ELISA)

Plate Trapped Antigen ELISA (PTA-ELISA) for detecting potyviruses SPFMV (Plate 2) and SPMMV were carried out for 37 *I. batatas* leaf samples selected from the whole sample collection showing different symptoms of virus infection. Respective positive samples gifted by DSMZ were used as positive controls and a healthy non host sample collected from the field was used as the

negative control. The readings were taken at 405 nm in a BIO-RAD iMark microplate Reader (USA) and the chart plotted (Figure 6 and Figure 7). ELISA results (Table 6) showing high positive values and symptoms were further screened with PCR. Out of 37 samples tested for SPFMV, 8 samples showed high OD value (above 1) which showed these samples were highly susceptible to SPFMV infection. These 8 samples were S1425, Sreebhadra, S758, S732, S420053, S18, S1504 and S1294 with common symptoms such as severe chlorotic spots with purple rings, feathering with purple borders and purple fill between the area of chlorotic spots. Rest of the samples were positive for SPFMV. For SPMMV infection, all samples were negative. Based on the ELISA reaction 32 samples were selected for the representative sample set which showed both high and moderate infection of SPFMV.

4.2.2 Dot Immuno Binding Assay (DIBA)

DIBA was carried out with 32 representative samples using SPFMV and SPMMV polyclonal antibodies acquired from DSMZ, Germany. 7 samples showed positive results for SPFMV (Plate 3) and all samples showed negative for SPMMV. Compared to ELISA, DIBA reaction was less and faint (Table 7); due to the low virus concentration in the sap extract taken; the sap extract used for DIBA being only 5 µl compared to 200 µl used in ELISA. The 7 samples showed positive were S1425, Sreebhadra, S758, S420053, S18, S1504 and S1294 with common symptoms such as severe chlorotic spots with purple rings, feathering with purple borders and purple fill between the area of chlorotic spots. Respective positive samples gifted by DSMZ were used as positive controls and a healthy non host sample collected from the field was used as the negative control.

ELISA was more sensitive than DIBA. 32 samples were positive for SPFMV in ELISA while 7 samples were positive for SPFMV in DIBA.

4.3 NUCLIEC ACID EXTRACTION

4.3.1 DNA isolation

CTAB (2 percent) protocol of DNA isolation was carried out using representative samples. The DNA of the 32 representative samples was isolated. To avoid protein contamination 2 g PVPP was added during grinding for every 80-100 mg of leaf tissue. The extracted genomic DNA was run on an agarose gel (1%) and visualized under UV to observe the bands. To quantify the DNA isolated spectrophotometric readings at 260 nm (OD_{260}) were taken and calculated using dilution factor. The dilution factor was 100 when 10 µl of DNA was made upto 1 ml. Based on the relationship that an A_{260} of 1.0 equals 50µg/ml pure DNA, the DNA was quantified. Purity of isolated DNA was calculated using OD_{260} and OD_{280} (Table 8). Amount of isolated DNA of 32 samples was between 390 µg/ml and 725 µg/ml and the purity was in the range of 1.6 to 1.8.

4.3.2 Isolation of total RNA

RNA isolation from representative 32 samples was carried out using LiCl protocol. The extracted RNA was run on an agarose gel (1.2%) and visualized under UV to observe the bands. To quantify the isolated RNA, spectrophotometric readings at 260 nm (OD_{260}) were taken and calculated using dilution factor (Table 9). The dilution factor was 100 when 10 µl of RNA was made upto 1 ml. Based on the relationship that an A_{260} of 1.0 equals 40µg/ml pure RNA, the RNA was quantified. Purity of isolated RNA was calculated using OD_{260} and OD_{280}. Amount of isolated RNA of 32 samples was between 396 µg/ml and 880 µg/ml and the purity was in the range of 1.9 to 2.2.

4.3.3 cDNA synthesis

The RNA isolated from the 32 samples was 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 AMV Reverse Transcriptase and oligo-dT primer. The synthesized cDNA was run on an agarose gel (1%) and visualized under UV to observe the bright sheared bands (Plate 4c).

4.4. 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 viruses infecting sweet potato viruses. Primers were got synthesized from Eurofins, India.

4.4.1 PCR for detection of potyviruses

Detection of *Potyvirus* infection was carried out using potyvirus group specific primers (Table 3) providing corresponding PCR conditions. A non template was used as control having all the components of a typical PCR but no template DNA. PCR was done for the representative 32 samples and four samples from Bhubaneswar for detection of potyviruses. Single band representing the amplicon size 188 bp and 800 bp for AtropaNad2.1a/AtropaNad2.2b (Thompson *et al.*, 2003), 350 bp for NIb2F/NIb3R (Zheng *et al.*, 2008), 1300 bp for Pot1/Pot2 (Colinet *et al.*, 1998), 327 bp for MJ1/MJ2 (Marie-Jeanne *et al.*, 2000), 719 bp for MJ1/M4T (Marie-Jeanne *et al.*, 2000), 1300 bp and 700 bp for Pot 1/Hrp-5 (Pappu *et al.*, 1998) was observed for virus positive 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. PCR results are given in Table 10 and gel electrophoresis images are shown in Plate 5.

4.4.2 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 regions in the genome of respective viruses.

4.4.2.1 PCR for detection of SPFMV

Detection of SPFMV infection in representative samples were carried out using SPFMV1 and SPFMV2 specific primers which amplifies the partial coat protein gene which gives 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 as mentioned in 3.4.3. An amplicon of size 411bp was observed as a single band for 29 virus positive samples in agarose gel (1%) electrophoresis (Plate 6a). No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR.

4.4.2.2 PCR for detection of SPMMV

Detection of SPMMV infection in representative samples were carried out using MMV1 and MMV2 specific primers which amplifies the partial coat protein gene which gives amplified product of 211 bp. A non template was used as control having all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.4. No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR. Out of 32 samples screened with MMV1 and MMV2 primers, no samples exhibited positive results in agarose gel (1%) electrophoresis.

4.4.2.3 PCR for detection of SPLCV

Detection of SPLCV infection in representative samples were carried out using LCV1 and LCV2 specific primers which amplifies the partial coat protein gene which gives amplified product of 446 bp. A non template was used as control having

all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.5. An amplicon of size 446 bp was observed as a single band for 15 virus positive samples in agarose gel (1%) electrophoresis (Plate 6b). No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR.

4.4.2.4 PCR for detection of SPCFV

Detection of SPCFV infection in representative samples were carried out using PMB-136 and PMB-14 specific primers which amplifies the 5' end of coat protein gene and gives amplified product of 715 bp. A non template was used as control having all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.8. No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR. Out of 32 samples screened with PMB-136 and PMB-14 primers, no samples exhibited positive results in agarose gel (1%) electrophoresis.

4.4.2.5 PCR for detection of SPV2

Detection of SPV_2 infection in representative samples were carried out using PMB-25a and PMB-26 specific primers which amplifies the 5' end of coat protein gene and gives amplified product of 698 bp. A non template was used as control having all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.9. No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR. Out of 32 samples screened with PMB-25a and PMB-26 primers, no samples exhibited positive results in agarose gel (1%) electrophoresis.

4.4.2.6 PCR for detection of SPVG

Detection of SPVG infection in representative samples were carried out using SPG-F_1 and SPFCG$_2$-R_2 primers which gives amplified product of 1191 bp. A non template was used as control having all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.6. No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR. Out of 32 samples screened with SPG-F_1 and SPFCG$_2$-R_2 primers, no samples exhibited positive results in agarose gel (1%) electrophoresis.

4.4.2.7 PCR for detection of SPVC

Detection of SPVC infection in representative samples were carried out using SPC-F_1 and SPFCG$_2$-R_2 primers which gives amplified product of 836 bp. A non template was used as control having all the components of a typical PCR but no template DNA. PCR conditions were as mentioned in 3.4.7. No amplification was observed in the non template control, which indicates there is no non-specific binding in PCR. Out of 32 samples screened with SPC-F_1 and SPFCG$_2$-R_2 primers, no samples exhibited positive results in agarose gel (1%) electrophoresis.

Out of 32 samples, 29 samples gave amplicon of 411 bp for SPFMV, 16 samples gave amplicon of 446 bp for SPLCV with the respective virus specific primers. All samples were negative in PCR for SPMMV, SPCFV, SPVG, SPVC and SPV2. 15 samples had mixed infections of SPFMV and SPLCV.

4.4.3 Cloning and sequencing

The gel elutes of S1294, S684 and S270 were proceeded with cloning for better sequencing results. The eluted amplified products where cloned 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 plates (plate 7). Around 100 to 150 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 (Plate 8).The recombinant clones were analysed by colony PCR uing respective primers (SPFMV1/SPFMV2, LCV1/LCV2 and Pot1/Hrp-5) under appropriate conditions and they were analysed in 1% agarose gel. Gel elution was carried out with one colony PCR positive clone each for SPFMV, SPLCV and SPVG. Gel eluted amplicons of size 411 bp, 446 bp and 1300 bp of respective samples S1294, S684 and S270 were sent to the Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB) with their respective primers. The sequencing results were obtained as electropherogram resulting from capillary sequencing in *.abi* format. The SPFMV sequence obtained is given below (304 nt):

CAAGATGAATGCAAATAAGAAAAGGCAACCAATGGTCAATGGAAGGGC
CATTATAAATTTCCAGCACCTATCAACATATGAACCAGAGCAGTTTGAGG
TTGCAAACACCCGTTCGACTCAAGAACAATTTCAAGCATGGTATGAAGG
AGTTAAAGGGGATTATGGTGTTGACGACACAGGAATGGGGATTTTAATG
AATGGACTAATGGTTTGGTGCATTGAAAATGGCACATCCCCAAATATAA
ATGGTGTGTGGACAATGATGGATGGTGATGAGCAAGTGACATATCCCAA
TTTAACCCTT.

The SPLCV sequence obtained is given below (418 nt):

TGTCCCGGTTTCAAGAGGCGTCCGGATGAAGAGAAAGAGGGGTGACCGC
ATCCCGAAGGGATGTGTCGGTCCCTGTAAGGTCCAGGACTATGAGTTCAA
GATGGACGTTCCCCACACGGGAACGTTTGTTTGTGTCTCGGATTTTACTA
GGGGGACTGGTCTTACCCATCGGCTGGGTAAGCGTGTTTGTGTGAAGTCC
ATGGGCATAGATGGGAAGGTATGGATGGATGACAATGTGGCGAAGAGA
GATCACACCAATATCATCACGTATTGGTTGCTTCGTGACAGAAGGCCCAA
CAAGGATCCGTTGAACTTTGGCCAGGTATTCACCATGTACGACAACGAGC
CCCCTACTGCTAAGATCCGAATGGATTTGAGGGATAGAATGCAGATCTTA
AAGGAAATT.

The SPVG sequence obtained is given below (251 nt):

AAATGCATAAAAATTTTATTAAAATCCTATTTAACAATCATTTACTCCTA
ATCTTCCCTGACACCCTCGTACCAAGCATACTTTGTTACTGTGTTGCTCGA
ATATTTGAAAGACTTGTCTGTTCCTGGTCATACATTGTCAGTGCTCAAGA
TTGACTACTATTCTTCCATTTGCCATTGGCGCTCTTTTCTTACTATGTGTTA
TCTGAAGCCGAGGCTCTATTATTCTCAACCGTCACTCCTTTTCCTTTT.

4.4.4 Sequence analysis

The sequence results were initially analysed and edited using BioEdit Sequence Alignment Editor program version 7.2.5 and the obtained sequence was run through the online BLAST program of NCBI.

BLAST Map of the query sequence (304 nt) gave 100 hits related to SPFMV similarity with reference to region 864-1208, accession number EU021070. The obtained 304 nt SPFMV sequence showed maximum similarity of 96% to *Sweet potato feathery mottle virus isolate Fe polyprotein gene, partial cds* (Figure 8).

BLAST Map of the query sequence (418 nt) gave 100 hits related to SPLCV similarity with reference to region 186-585, accession number KF475971. The obtained 418 nt SPLCV sequence showed maximum similarity of 96% to *Sweet potato leaf curl virus strain China: CHUAN16:2012 coat protein gene, complete cds* and *Sweet potato leaf curl isolate CTCRI TVM M1, complete genome* (Accession KM050768) (Figure 9).

BLAST Map of the query sequence (251 nt) gave 100 hits related to SPVG similarity with reference to region 9887 -10090 accession number KM014815. The obtained 251 nt SPVG sequence showed maximum similarity of 90% to Sweet *potato virus G isolate IS103, complete genome* (Figure 10).

Phylogenic tree was constructed with similar sequences using online NCBI blast analysis software. The trees constructed at 100 bootstrap replicates showed similarity with the Fe polyprotein gene region of SPFMV isolates (Figure 11), CP region of SPLCV isolates (Figure 12) and complete genome of SPVG isolates (Figure 13) respectively. Phylogenetic analysis clearly revealed that the sequences obtained in this study belongs to SPFMV for sample S1294, SPLCV for sample S684 and SPVG for sample S270 as they grouped along with their respective virus sequences used for comparison analysis.

After the both serological and nucleic acid based screening, 29 samples among representative sample set showed SPFMV infection and 16 samples showed SPLCV infection. About 15 samples showed mixed infection of SPFMV and SPLCV. One of the samples co-infected with SPFMV and SPLCV was also infected with SPVG. There was no infection for SPMMV, SPVC, SPV2 and SPCFV. The combined test results of all the methods executed for virus detection is represented in the Table 12.

Table 5: Representative sample set, location and symptoms observed

Sl No.	Sample ID	Place of collection	Symptoms
1	S1425	CTCRI	Chlorotic spots, purple fill between the area of chlorotic spots
2	SREE BHADRA	CTCRI	Separated chlorotic spots with purple rings
3	S1294	CTCRI	Severe purple fill between the area of chlorotic spots
4	S1364	CTCRI	Mild chlorotic spots without purple rings
5	S732	CTCRI	Feathering with purple borders, purple fill between the area of chlorotic spots
6	S1073	CTCRI	Mild yellow veining
7	S837	CTCRI	Mild feathering
8	S684	CTCRI	Severe upward curling, mild purpling around chlorotic spots
9	S758	CTCRI	Severe chlorotic spots, purple fill between the area of chlorotic spots
10	S658	CTCRI	Feathering with purple borders
11	S275	CTCRI	Mild feathering, dark purple fill between the area of chlorotic spots
12	S478	CTCRI	Mild puckering, purple chlorotic spots
13	S270	CTCRI	Severe feathering with dark purple borders
14	S219	CTCRI	Separated chlorotic spots with purple rings
15	S717/1	CTCRI	Severe feathering with dark purple borders
16	S175	CTCRI	Mild puckering
17	S68	CTCRI	Chlorotic spots without purple rings
18	SA1	CTCRI	Mild puckering, chlorotic spots without purple rings
19	S1132	CTCRI	Feathering, chlorotic spots with purple rings
20	S188	CTCRI	Puckering, mild upward curling

Table 5 (continued): Representative sample set, location and symptoms observed

Sl No.	Sample ID	Place of collection	Symptoms
21	S1322	CTCRI	Mild puckering, yellow netting
22	S570	CTCRI	Yellow netting, mild upward curling
23	S1504	CTCRI	Feathering, dark purple fill between the area of chlorotic spots
24	S1026	CTCRI	Mild puckering, chlorotic spots without purple rings
25	SV 27/5	CTCRI	Mild puckering
26	SHILLONG 1	CTCRI	Mild puckering
27	SREENI 1	CTCRI	Chlorotic spots without purple rings
28	S1521	CTCRI	Chlorotic spots with purple rings
29	S420053	CTCRI	Severe feathering, dark purple fill between the area of chlorotic spots
30	S1498	CTCRI	Chlorotic spots with purple rings
31	S14	CTCRI	Feathering, Chlorotic spots without purple rings
32	S18	CTCRI	Prominent, dark purple chlorotic spots

Table 6: ELISA readings of the samples using specific polyclonal antibody for SPFMV and SPMMV

Sl. No.	Samples	A_{405} Reading		ELISA	
		SPFMV	SPMMV	SPFMV	SPMMV
1	S275	0.483	- 0.058	+	-
2	S270	0.498	-0.293	+	-
3	S1073	0.111	-0.141	+	-
4	S1364	0.118	-0.462	+	-
5	S837	0.121	-0.396	+	-
6	S188	0.139	-0.469	+	-
7	S1425	1.079	-0.452	+++	-
8	Sreebhadra	1.392	-0.096	+++	-
9	S758	1.433	-0.069	+++	-
10	S684	0.143	-0.258	+	-
11	S717/1	0.792	0.308	++	-
12	S1132	0.508	-0.269	++	-
13	S14	0.107	-0.301	+	-
14	SA1	0.281	-0.396	+	-
15	S478	0.125	-0.469	+	-
16	175	0.101	-0.008	+	-
17	S68	0.114	-0.269	+	-
18	S1026	0.115	-0.301	+	-
19	S732	1.402	-0.258	+++	-
20	420053	1.466	-0.214	+++	-
21	S570	0.317	-0.346	+	-

Table 6 (continued): ELISA reading of the samples using specific polyclonal antibody for SPFMV and SPMMV

Sl. No.	Samples	A_{405} Reading		ELISA	
		SPFMV	SPMMV	SPFMV	SPMMV
22	S658	0.562	-0.346	++	-
23	Shillong 1	0.091	-0.102	+	-
24	Sreeni 1	0.056	-0.269	+	-
25	S1521	0.516	-0.2	++	-
26	S1322	0.137	-0.008	+	-
27	S219	0.529	-0.269	++	-
28	S1498	0.623	-0.043	++	-
29	S18	1.388	-0.102	+++	-
30	S1504	1.619	-0.2	++++	-
31	S1294	1.693	-0.043	++++	-
32	SV 27/5	0.086	-0.143	+	-
33	SAMPLE 38	0.044	-0.036	+	-
34	SAMPLE 39	0.031	-0.058	+	-
35	SAMPLE 40	0.124	-0.293	+	-
36	SAMPLE 41	0.021	-0.141	+	-
37	SAMPLE 42	0.043	-0.008	+	-
38	POSITIVE CONTROL	2.384	0.429	++++	+
39	NEGATIVE CONTROL	-0.011	-0.015	-	-

Note: + $0 \leq 0.5$

++ $0.5 \leq 1$

+++ $1 \leq 1.5$

++++ ≤ 1.5

Table 7: Reaction of samples to DIBA

Sr. No.	Samples	Reaction		Sr. No.	Samples	Reaction	
		SPFMV	SPMMV			SPFMV	SPMMV
1	S275	-	-	21	S570	-	-
2	S270	-	-	22	S658	-	-
3	S1073	-	-	23	**Positive Control**	+	+
4	S1364	-	-	24	Shillong 1	-	-
5	S837	-	-	25	Sreeni 1	-	-
6	S188	-	-	26	S1521	-	-
7	S1425	+	-	27	S1322	-	-
8	Sreebhadra	+	-	28	S219	-	-
9	S758	+	-	29	S1498	-	-
10	S684	-	-	30	S18	+	-
11	S717/1	-	-	31	S1504	+	-
12	SS1132	-	-	32	S1294	+	-
13	S14	-	-	33	SVS 27/5	-	-
14	SA1	-	-	34	SAMPLE 38	-	-
15	S478	-	-	35	Negative Control	-	-
16	S175	-	-	36	**Buffer Control**		
17	S68	-	-	37	**Buffer Control**		
18	S1026	-	-	38	**Buffer Control**		
19	S732	-	-	39	**Buffer Control**		
20	S420053	+	-	40	**Buffer Control**		

Table 8: Quantification of DNA of representative set isolated using spectrophotometric readings

Sr. No.	Samples	A_{260}	A_{280}	Purity (A_{260}/A_{280})	Amt. of DNA(µg/ml)
1	S275	0.122	0.071	1.71	610
2	S270	0.091	0.055	1.65	455
3	S1073	0.098	0.058	1.68	490
4	S1364	0.130	0.075	1.73	650
5	S837	0.125	0.069	1.81	625
6	S188	0.111	0.061	1.81	555
7	S1425	0.082	0.049	1.67	410
8	Sreebhadra	0.097	0.060	1.61	485
9	S758	0.086	0.048	1.60	385
10	S684	0.141	0.081	1.74	705
11	S717/1	0.115	0.067	1.71	575
12	S1132	0.097	0.059	1.64	485
13	S14	0.111	0.068	1.63	555
14	SA1	0.142	0.081	1.75	710
15	S478	0.102	0.063	1.61	510
16	S175	0.089	0.050	1.78	445
17	S68	0.131	0.074	1.77	655
18	S1026	0.090	0.054	1.66	450
19	S732	0.078	0.046	1.69	390
20	S420053	0.082	0.049	1.67	410
21	S570	0.088	0.051	1.72	440
22	S658	0.077	0.048	1.60	385
23	Shillong 1	0.087	0.054	1.61	435
24	Sreeni 1	0.087	0.054	1.61	435
25	S1521	0.088	0.051	1.72	440
26	S1322	0.099	0.059	1.67	495
27	S219	0.094	0.058	1.62	470
28	S1498	0.095	0.059	1.61	475
29	S18	0.121	0.069	1.75	605
30	S1504	0.101	0.059	1.71	505
31	S1294	0.110	0.063	1.74	550
32	SV 27/5	0.145	0.084	1.72	725

Table 9: Quantification of RNA of representative set isolated using spectrophotometric readings

Sr. No.	Samples	A_{260}	A_{280}	Purity (A_{260}/A_{280})	Amt. of RNA(µg/ml)
1	S275	0.20	0.098	2.04	800
2	S270	0.135	0.061	2.21	540
3	S1073	0.141	0.069	2.04	564
4	S1364	0.119	0.057	2.08	476
5	S837	0.199	0.099	2.01	796
6	S188	0.129	0.062	2.08	516
7	S1425	0.183	0.091	2.01	732
8	Sreebhadra	0.116	0.059	1.96	464
9	S758	0.176	0.081	2.17	704
10	S684	0.130	0.063	2.06	520
11	S717/1	0.127	0.059	2.15	508
12	S1132	0.119	0.057	2.08	476
13	S14	0.115	0.059	1.94	460
14	SA1	0.150	0.073	2.05	600
15	S478	0.187	0.082	2.28	748
16	S175	0.133	0.066	2.01	532
17	S68	0.136	0.068	2.0	544
18	S1026	0.099	0.051	1.94	396
19	S732	0.188	0.085	2.21	752
20	S420053	0.149	0.071	2.09	596
21	S570	0.022	0.10	2.20	880
22	S658	0.161	0.077	2.09	644
23	Shillong 1	0.144	0.063	2.28	576
24	Sreeni 1	0.116	0.06	1.93	464
25	S1521	0.195	0.089	2.19	780
26	S1322	0.148	0.065	2.27	592
27	S219	0.138	0.063	2.19	552
28	S1498	0.197	0.091	2.16	788
29	S18	0.21	0.098	2.14	840
30	S1504	0.139	0.062	2.24	556
31	S1294	0.147	0.071	2.07	588
32	SV 27/5	0.131	0.064	2.04	524

Table 10: Screening of sweet potato samples for virus infection using potyvirus group specific primers

Sr. No.	Samples	AtropaNad2. 1a/2b	Nlb2 F/3R	CN48/ Oligo-dT	Pot 1/Pot2	Hrp-5/ Oligo-dT	MJ1/ MJ2	MJ1/ M4T	Pot 1/ Hrp-5
1	S275	-	-	-	-	-	✓	-	-
2	S270	-	-	-	-	-	✓	-	✓
3	S1073	-	-	-	-	-	-	-	-
4	S1364	-	-	-	-	-	✓	-	-
5	S837	-	-	-	-	-	✓	-	✓
6	S188	-	-	-	-	-	✓	-	-
7	S1425	✓	✓	-	-	-	✓	-	✓
8	Sreebhadra	✓	✓	-	-	-	✓	-	✓
9	S758	✓	✓	-	-	-	✓	-	-
10	S684	-	-	-	-	-	✓	-	-
11	S717/1	-	✓	-	-	-	✓	-	-
12	S1132	-	-	-	-	-	✓	-	-
13	S14	-	-	-	-	-	✓	-	✓
14	SA1	-	-	-	-	-	✓	-	✓
15	S478	-	-	-	-	-	✓	-	-
16	S175	-	✓	-	-	-	-	-	-
17	S68	-	-	-	-	-	-	-	-
18	S1026	-	-	-	-	-	✓	-	-
19	S732	-	-	-	✓	-	✓	-	✓
20	S420053	✓	✓	-	✓	-	✓	✓	-
21	S570	-	✓	-	-	-	✓	-	✓
22	S658	-	✓	-	-	-	✓	-	✓
23	Shillong 1	-	-	-	-	-	-	-	-

Table 10 (continued): Screening of sweet potato samples for virus infection using potyvirus group specific primers

Sr. No.	Samples	AtropaNad2. 1a/2b	Nlb2 F/3R	CN48/ Oligo-dT	Pot1/ Pot2	Hrp-5/ Oligo-dT	MJ1/ MJ2	MJ1/ M4T	Pot 1/ Hrp-5
24	Sreeni 1	-	-	-	-	-	✓	-	✓
25	S1521	-	-	-	-	-	✓	-	-
26	S1322	-	-	-	-	-	✓	-	-
27	S219	-	-	-	-	-	✓	-	-
28	S1498	-	✓	-	-	-	✓	-	-
29	S18	-	✓	-	✓	-	✓	-	-
30	S1504	✓	✓	-	-	-	✓	-	✓
31	S1294	✓	✓	-	✓	-	✓	-	✓
32	SV 27/5	-	-	-	-	-	-	-	-

Table 11: Screening of sweet potato samples for virus infection using virus specific primers

Sr. No.	Samples	SPFMV1/ SPFMV2	MMV1/ MMV2	LCV1/ LCV2	PMB-136/ PMB-14	PMB-25a/ PMB-26	SPG-F1/ SPFCG2-R2	SPC-F1/ SPFCG2-R2
1	S275	✓	-	✓	-	-	-	-
2	S270	✓	-	✓	-	-	-	-
3	S1073	✓	-	✓	-	-	-	-
4	S1364	✓	-	✓	-	-	-	-
5	S837	✓	-	-	-	-	-	-
6	S188	✓	-	✓	-	-	-	-
7	S1425	✓	-	✓	-	-	-	-
8	Sree bhadra	✓	-	✓	-	-	-	-
9	S758	✓	-	-	-	-	-	-
10	S684	✓	-	✓	-	-	-	-
11	S717/1	✓	-	-	-	-	-	-
12	S1132	✓	-	-	-	-	-	-
13	S14	✓	-	-	-	-	-	-
14	SA1	✓	-	-	-	-	-	-
15	S478	✓	-	-	-	-	-	-
16	S175	✓	-	-	-	-	-	-
17	S68	-	-	-	-	-	-	-
18	S1026	✓	-	✓	-	-	-	-
19	S732	✓	-	✓	-	-	-	-
20	S420053	✓	-	-	-	-	-	-
21	S570	✓	-	-	-	-	-	-
22	S658	✓	-	✓	-	-	-	-
23	Shillong 1	-	-	✓	-	-	-	-
24	SREENI 1	✓	-	✓	-	-	-	-
25	S1521	✓	-	-	-	-	-	-
26	S1322	✓	-	-	-	-	-	-
27	S219	✓	-	-	-	-	-	-
28	S1498	✓	-	✓	-	-	-	-
29	S18	✓	-	✓	-	-	-	-

Table 11 (continued): Screening of sweet potato samples for virus infection using virus specific primers

Sr. No.	Samples	SPFMV1/ SPFMV2	MMV1/ MMV2	LCV1/ LCV2	PMB-136/ PMB-14	PMB-25a/ PMB-26	SPG-F1/ SPFCG2-R2	SPC-F1/ SPFCG2-R2
30	S1504	✓	-	-	-	-	-	-
31	S1294	✓	-	✓	-	-	-	-
32	SV 27/5	-	-	-	-	-	-	-

Table 12: Sample reactions for each test to detect virus infection

Sample	ELISA	DIBA	PCR						
	SPFMV	SPFMV	SPFMV	SPMMV	SPLCV	SPCFV	SPVG	SPVC	SPV2
S275	✓	-	✓	-	✓	-	-	-	-
S270	✓	-	✓	-	✓	-	✓	-	-
S1073	✓	-	✓	-	✓	-	-	-	-
S1364	✓	-	✓	-	✓	-	-	-	-
S837	✓	-	✓	-	-	-	-	-	-
S188	✓	-	✓	-	✓	-	-	-	-
S1425	✓	+	✓	-	✓	-	-	-	-
Sreebhadra	✓	+	✓	-	✓	-	-	-	-
S758	✓	+	✓	-	-	-	-	-	-
S684	✓	-	✓	-	✓	-	-	-	-
S717/1	✓	-	✓	-	-	-	-	-	-
S1132	✓	-	✓	-	-	-	-	-	-
S14	✓	-	✓	-	-	-	-	-	-
SA1	✓	-	✓	-	-	-	-	-	-
S478	✓	-	✓	-	-	-	-	-	-
S175	✓	-	✓	-	-	-	-	-	-
S68	✓	-	-	-	-	-	-	-	-
S1026	✓	-	✓	-	✓	-	-	-	-
S732	✓	-	✓	-	✓	-	-	-	-
S420053	✓	+	✓	-	-	-	-	-	-
S570	✓	-	✓	-	-	-	-	-	-
S658	✓	-	✓	-	✓	-	-	-	-

Table 12 (continued): Sample reactions for each test to detect virus infection

Sample	ELISA SPFMV	DIBA SPFMV	PCR						
			SPFMV	SPMMV	SPLCV	SPCFV	SPVG	SPVC	SPV2
Shillong 1	✓	-	-	-	✓	-	-	-	-
Sreeni 1	✓	-	✓	-	✓	-	-	-	-
S1521	✓	-	✓	-	-	-	-	-	-
S1322	✓	-	✓	-	-	-	-	-	-
S219	✓	-	✓	-	-	-	-	-	-
S1498	✓	-	✓	-	✓	-	-	-	-
S18	✓	+	✓	-	✓	-	-	-	-
S1504	✓	+	✓	-	-	-	-	-	-
S1294	✓	+	✓	-	✓	-	-	-	-
SV 27/5	✓	-	-	-	-	-	-	-	-

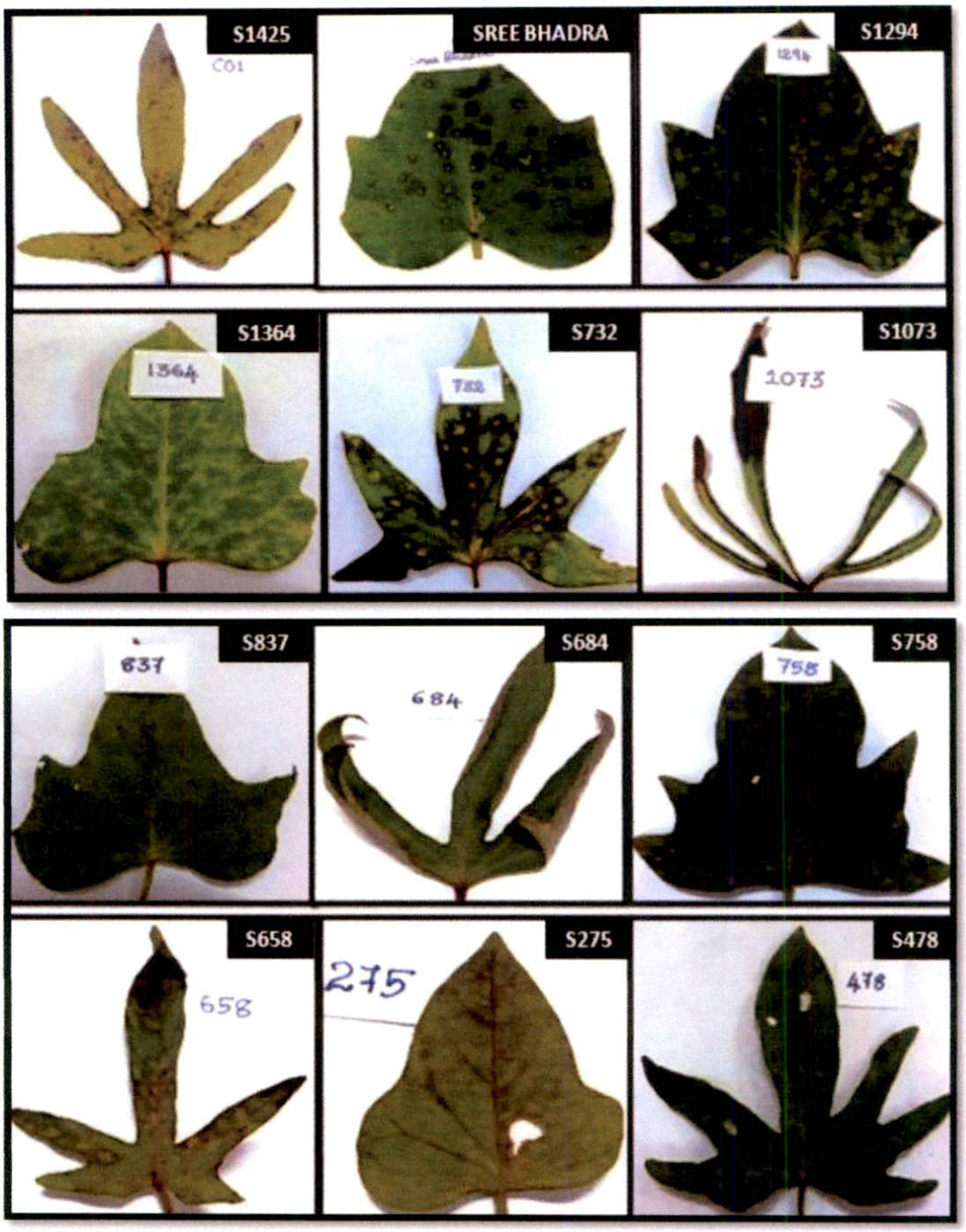


Plate 1: Representative sample set (Symptoms detail in Table 5)

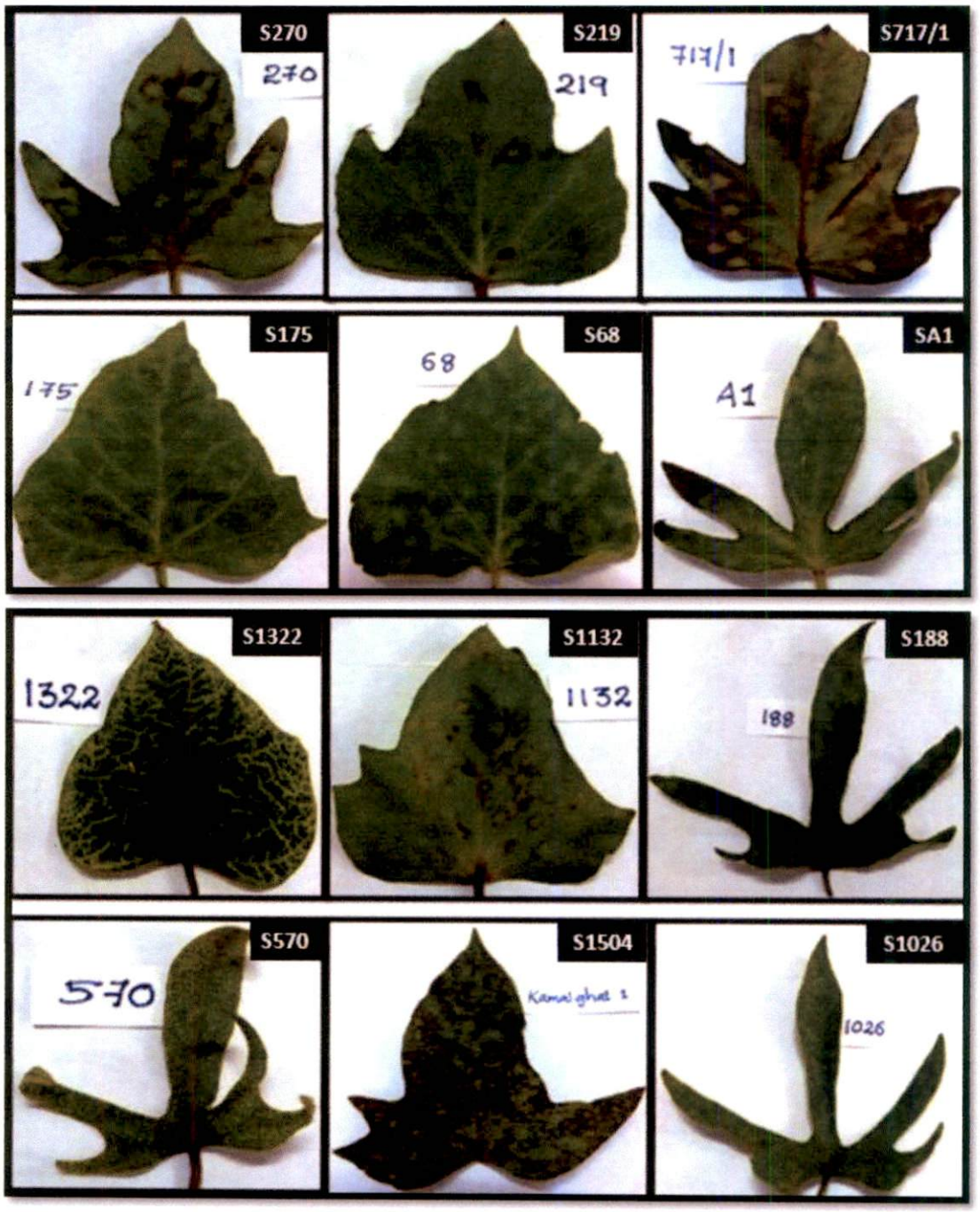


Plate 1(continued): Representative sample set (Symptoms detail in Table 5)

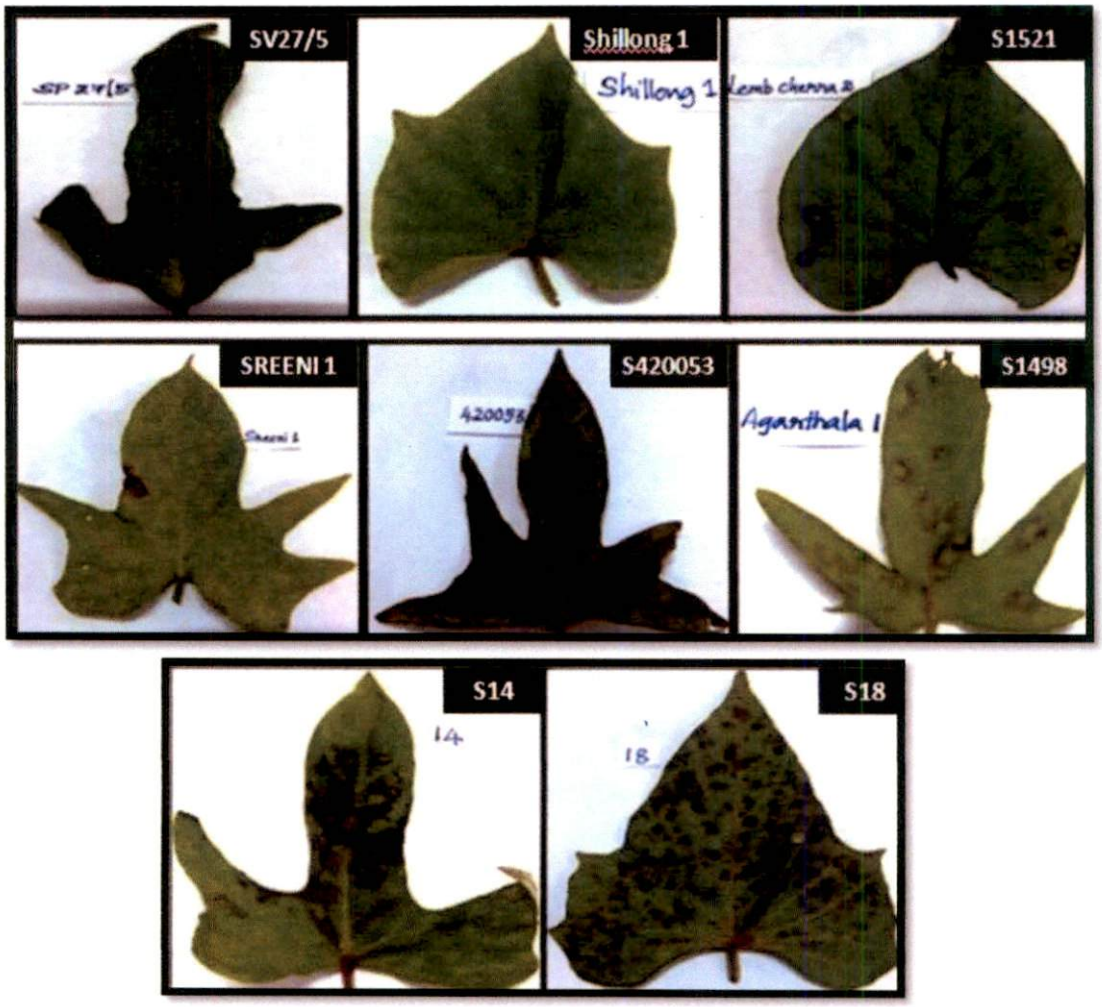


Plate 1(continued): Representative sample set (Symptoms detail in Table 5)

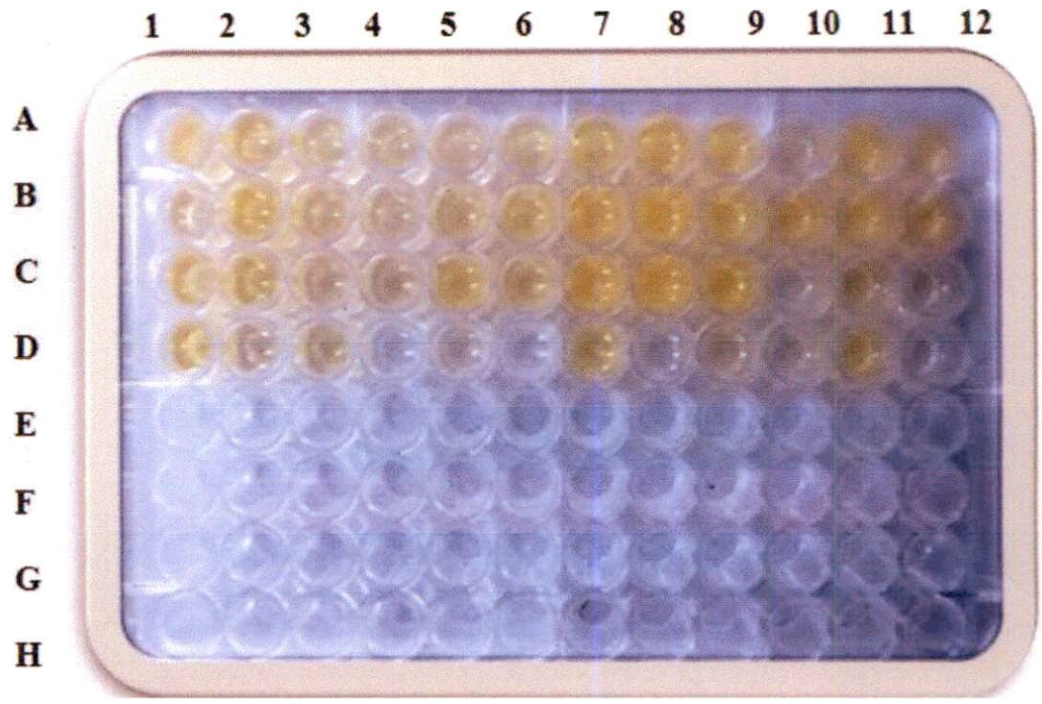


Plate 2: Serological analysis of representative samples using SPFMV ELISA

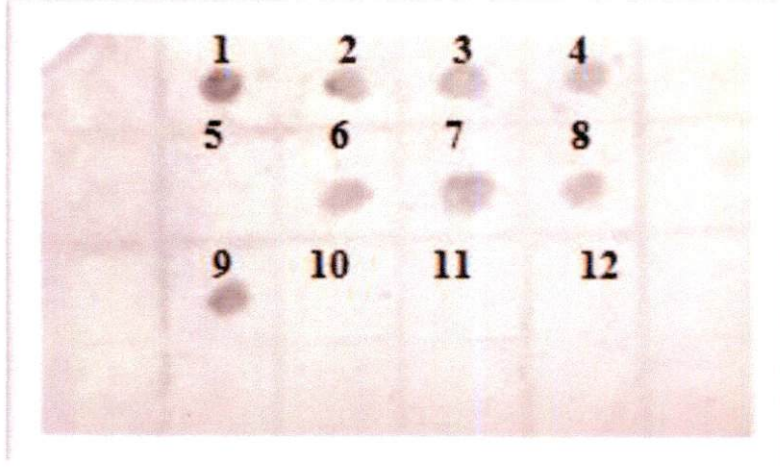


Plate 3: Samples showing positive in SPFMV DIBA

1.Positive Control, **2**. S1425, **3**. Sreebhadra, **4**. S758, **5**. Negative Control, **6**. S420053, 7. S18, **8**. S1504, **9**. S1294, **10, 11, 12**. Buffer control

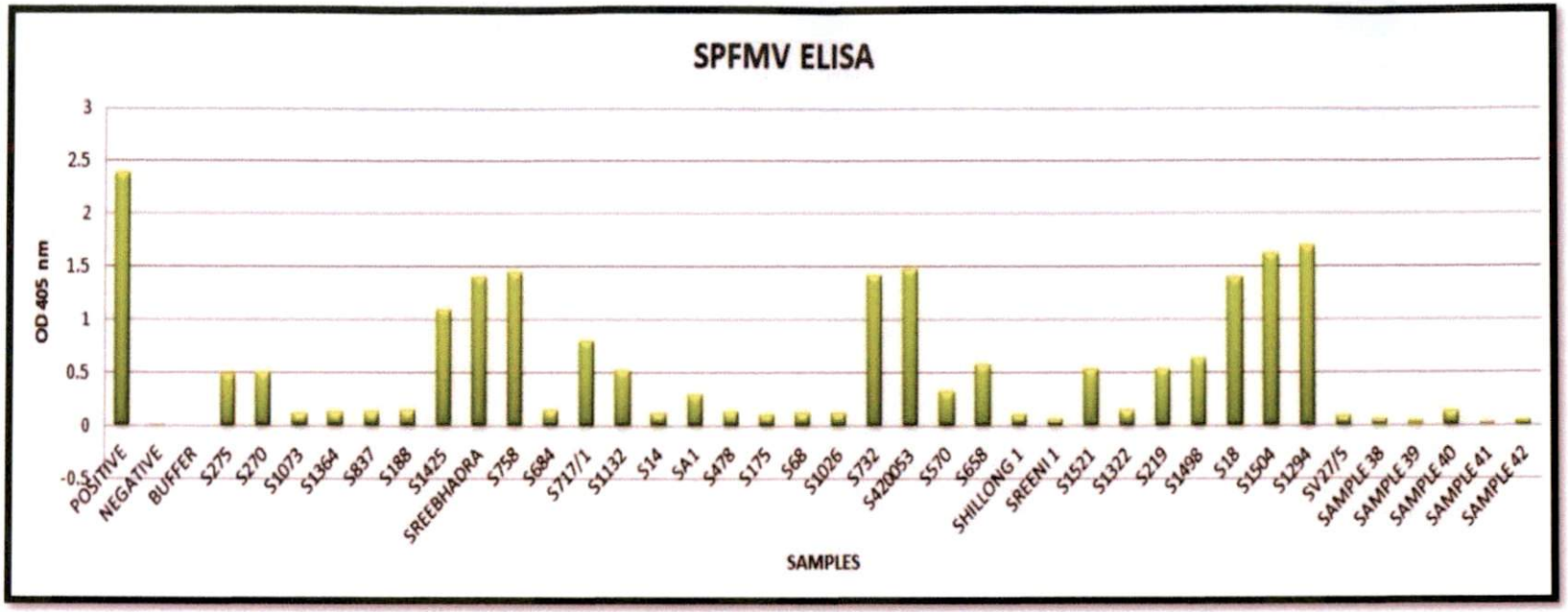


Figure 6: ELISA reactions for SPFMV of samples at 405 nm

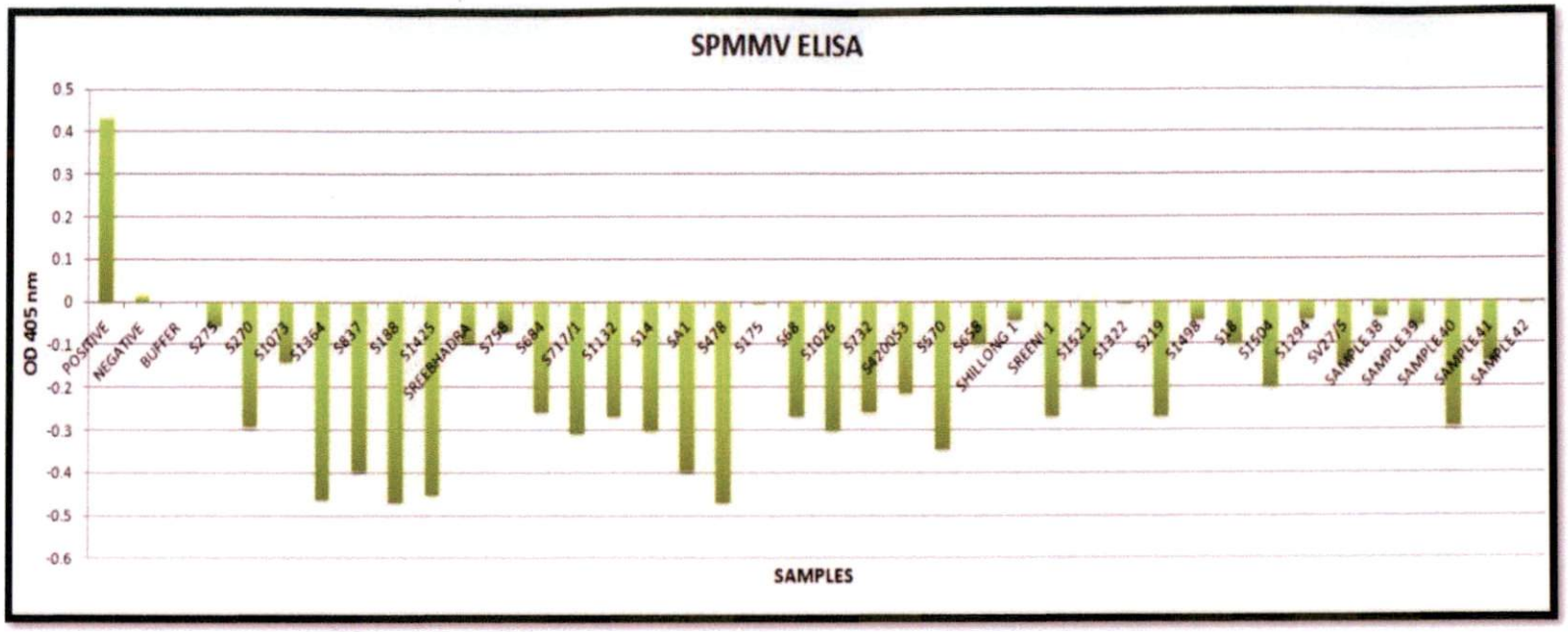


Figure 7: ELISA reactions for SPMMV of samples at 405 nm

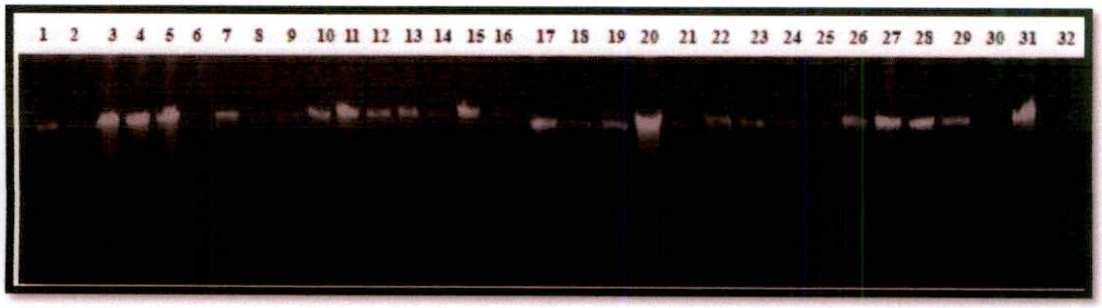


Plate (4a): Gel profile of DNA isolated by CTAB (2%) protocol

1-S1425, 2-S732, 3-S684, 4-SA1, 5-S68, 6-S658, 7-SREE BHADRA, 8-S420053, 9-SREENI 1, 10-S717/1, 11-S275, 12-S1322, 13-S1132, 14-S758, 15-S18, 16-SHILLONG 1, 17-S14, 18-S1498, 19-S1504, 20-S725, 21-S1521, 22-S219, 23-S270, 24-S570, 25-S175, 26-S478, 27-S1294, 28-S837, 29-S188, 30-S1026, 31-

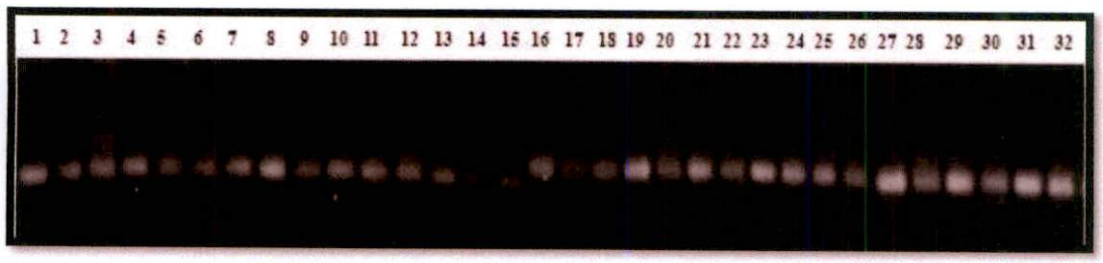


Plate (4b): Gel profile of RNA isolated by LiCl method

1-S1425, **2**-S732, **3**-S684, **4**-SA1, **5**-S68, **6**-S658, **7**-SREE BHADRA, **8**-S420053, **9**-SREENI 1, **10**-S717/1, **11**-S275, **12**-S1322, **13**-S1132, **14**-S758, **15**-S18, **16**-SHILLONG 1, **17**-S14, **18**-S1498, **19**-S1504, **20**-S725, **21**-S1521, **22-S**219, **23-S**270, **24-S**570, **25**-S175, **26**-S478, **27**-S1294, **28**-S837, **29**-S188, **30**-S1026, **31**-S1364, **32**-S1073

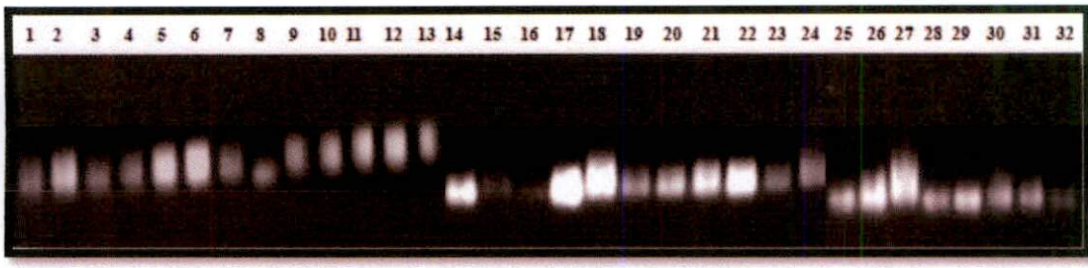


Plate (4c): Gel profile of cDNA

1-S1425, **2**-S732, **3**-S684, **4**-SA1, **5**-S68, **6**-S658, **7**-SREE BHADRA, **8**-S420053, **9**-SREENI 1, **10**-S717/1, **11**-S275, **12**-S1322, **13**-S1132, **14**-S758, **15**-S18, **16**-SHILLONG 1, **17**-S14, **18**-S1498, **19**-S1504, **20**-S725, **21**-S1521, **22**-S219, **23**-S270, **24**-S570, **25**-S175, **26**-S478, **27**-S1294, **28**-S837, **29**-S188, **30**-S1026, **31**-S1364, **32**-S1073

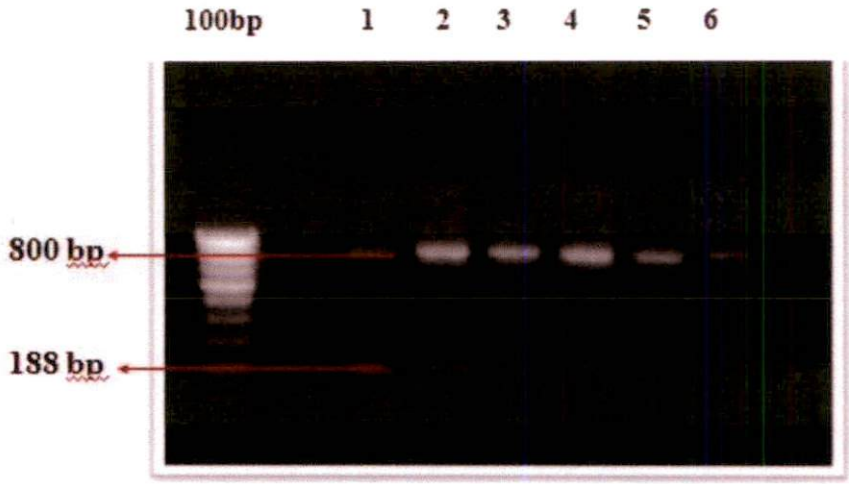


Plate 5(a) Agarose gel electrophoresis of sweet potato samples positive for AtropaNad2.1a/2b primers

1.S1425, **2**.Sreebhadra, **3**.S1294, **4**.S758, **5**.S1504, **6**.S420053

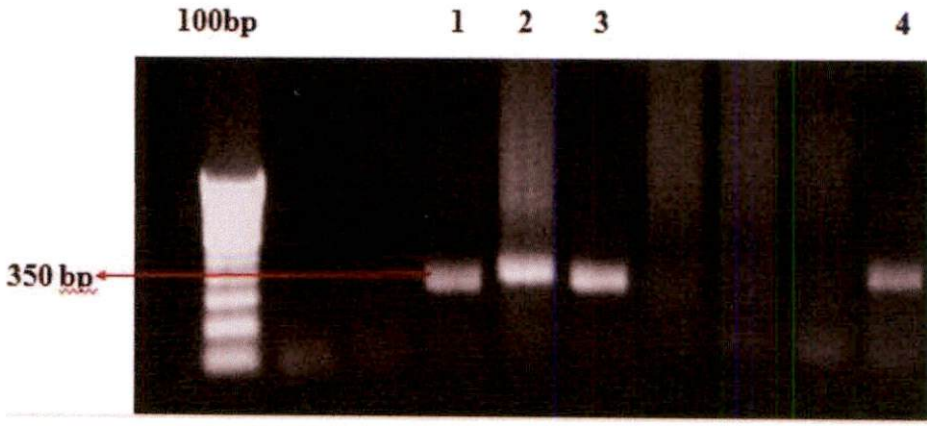


Plate 5(b) Agarose gel electrophoresis of sweet potato samples positive for NIb2F/3R primers

1-S1425, **2**-S1294, **3**-S758, **4**-S570

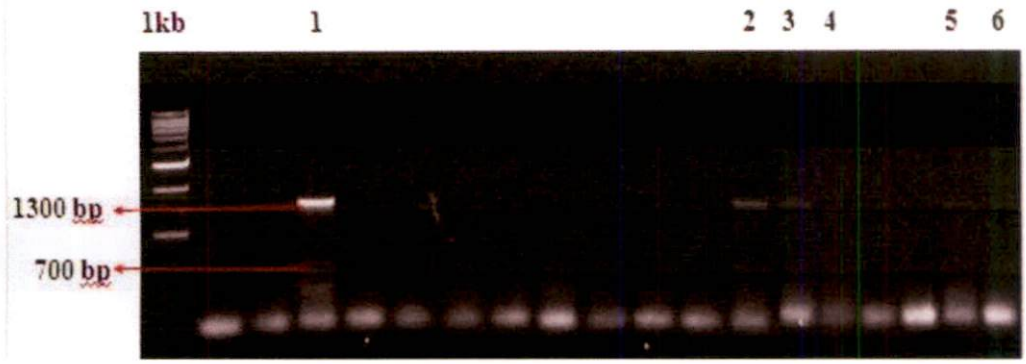


Plate 5(c) Agarose gel electrophoresis of sweet potato samples positive for Pot1/Hrp-5 primers

1-S270, **2**-SREE BHADRA, **3**-S570, **4**-S1504 **5**-S732, **6**-SA1

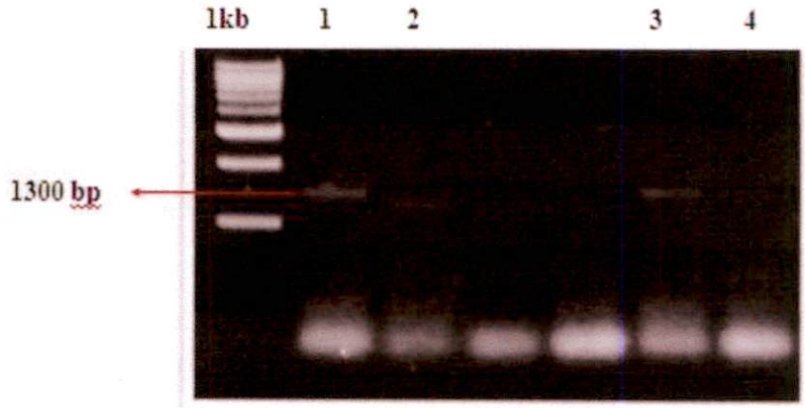


Plate 5(d) Agarose gel electrophoresis of sweet potato samples positive for Pot1/2 primers

1- S1294, **2**-S732, **3**-S420053, **4**-S18

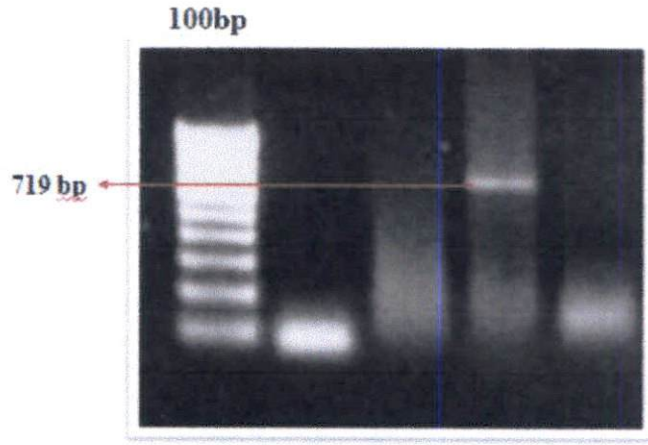


Plate 5(e) Agarose gel electrophoresis of sweet potato samples positive for MJ1/M4T primers

S420053

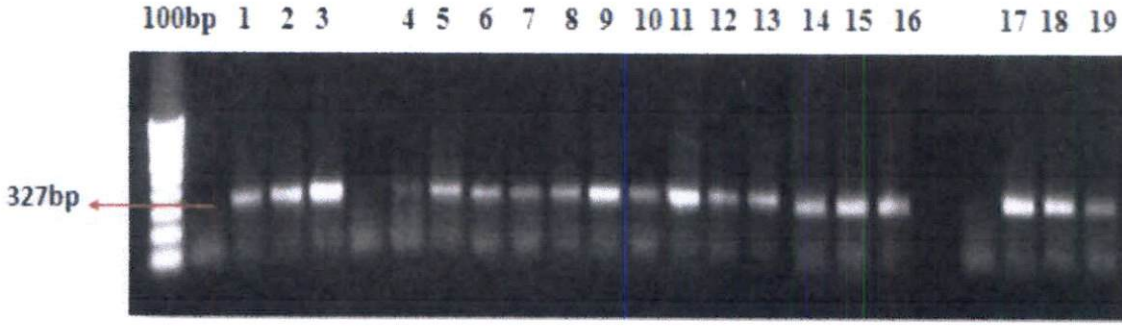


Plate 5(f) Agarose gel electrophoresis of sweet potato samples positive for MJ1/MJ2 primers

1-S837, **2**-S717/1, **3**-S1294, **4**-S219, **5**-S1521, **6**-SREE BHADRA, **7**-S1364, **8**-S732, **9**-S758, **10**-S1498, **11**-S420053, **12**-S658, **13**-S270, **14**-S188, **15**-S1504, **16**-S18, **17**-S1425, **18**-S570, **19**-S275

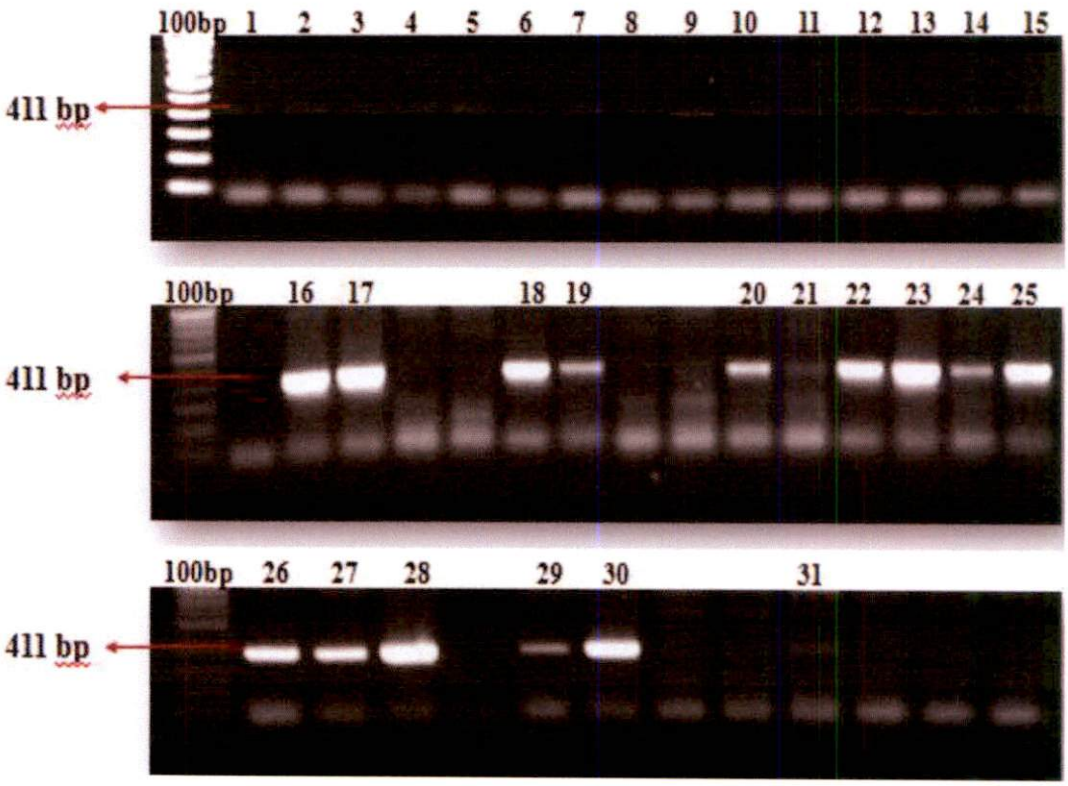


Plate 6(a) Agarose gel electrophoresis of sweet potato samples positive for SPFMV1/SPFMV2 primers

1.S1364, **2**.S1073, **3**.S837, **4**.S684, **5**.S658, **6.**S275, **7.**S478, **8**.S219, **9**.S717/1, **10.**S175, **11**.SA1, **12**.S1132, **13**.S188, **14**.S1322, **15**.S1026, **16**.S1294, **17**.758, **18**.S18, **19**.Sreeni1, **20**.S570, **21**.S270, **22**.S1521, **23**.S1504, **24**.S1425, **25**.S732, **26**.Sreebhadra, **27**.S14, **28**.S420053, **29**. Bhubaneswar 2, **30**.S1498, **31**. Bhubaneswar1

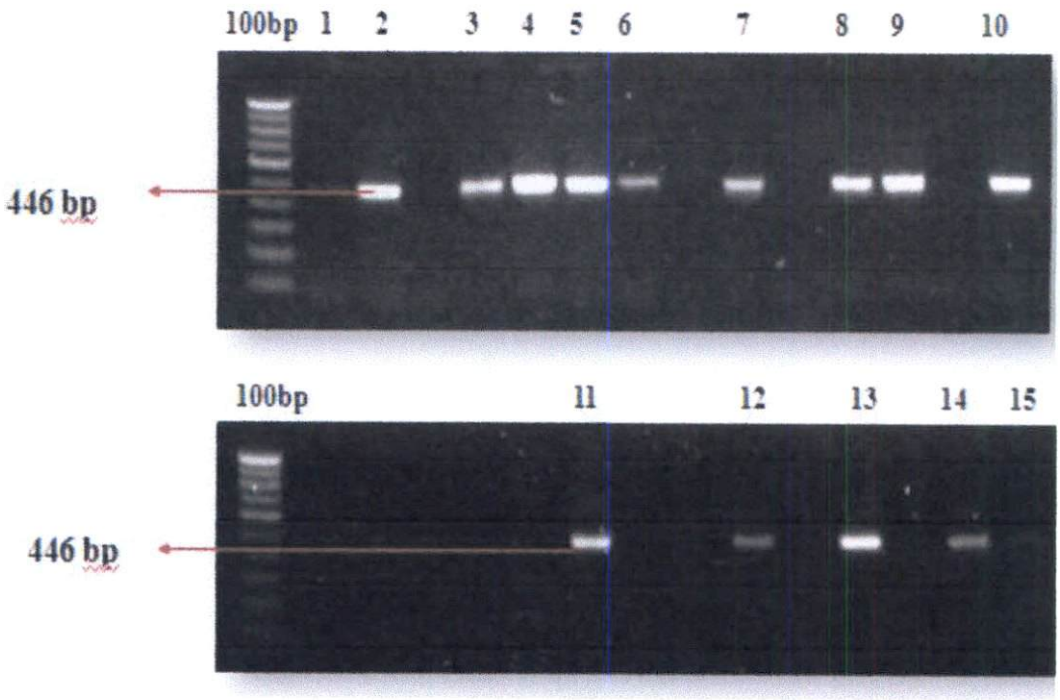


Plate 6(b) Agarose gel electrophoresis of sweet potato samples positive for LCV1/LCV2 primers

1-S1425, **2**-SREE BHADRA, **3**-S1294, **4**-S684, **5**-S188, **6**-S1364, **7**-S732, **8**-SREENI 1, **9**-S1026, **10**-S658, **11**-S1073, **12**-S275, **13**-S270, **14**-SHILLONG 1, **15**-S18

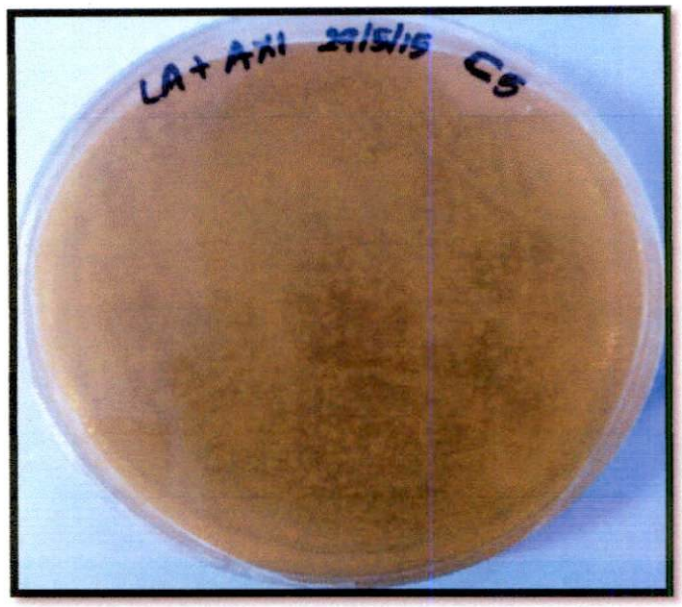


Plate 7 (a): Blue-white colony screening in LB-AXI plates

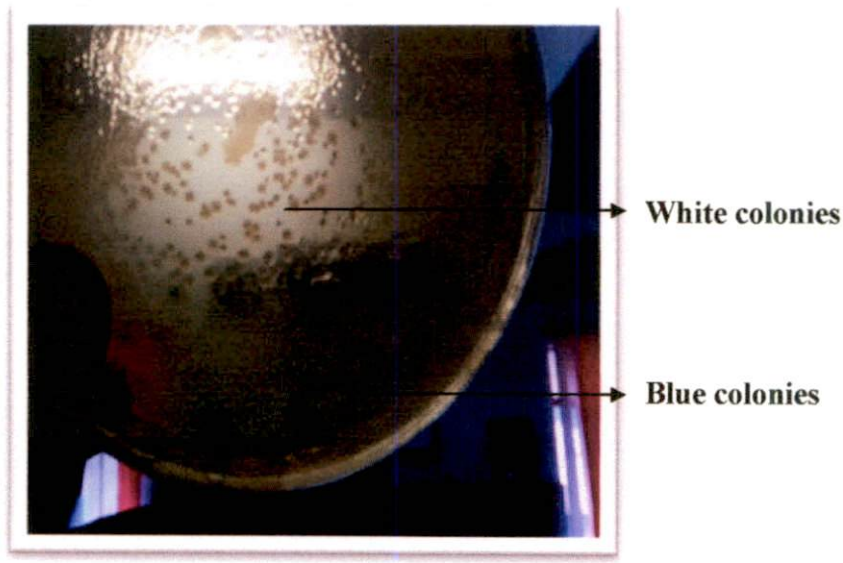


Plate 7(b): Blue and white colonies on LB-AXI plates

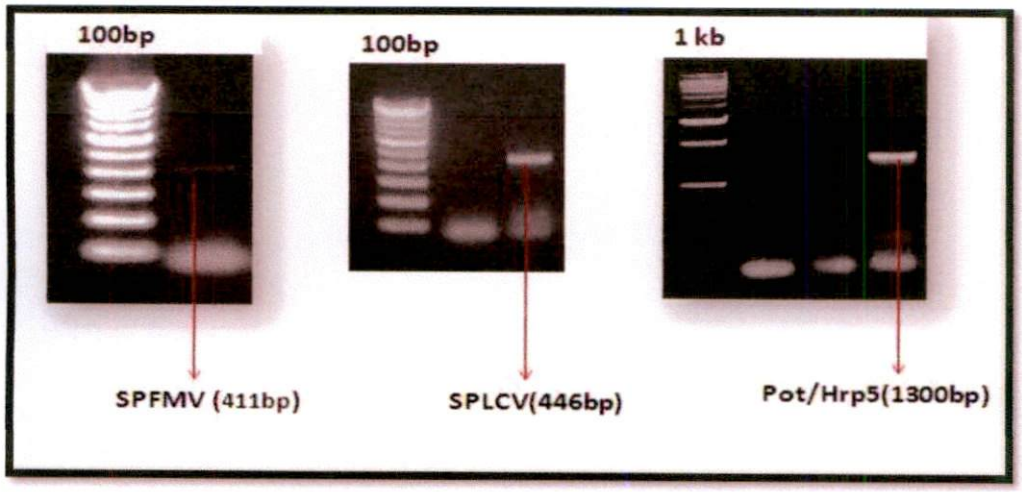


Plate 8: Colony PCR to confirm insert of amplicon

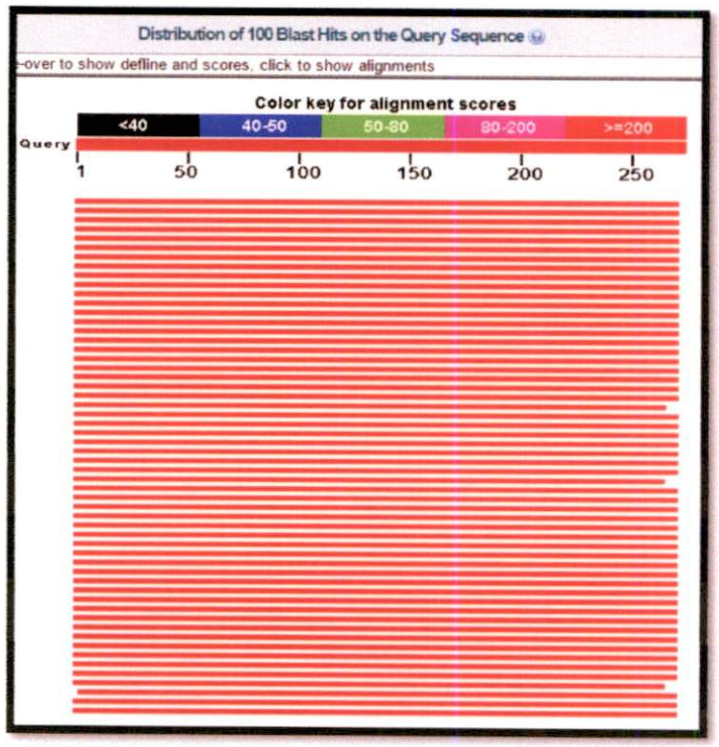


Figure 8: BLAST analysis of the SPFMV sequence

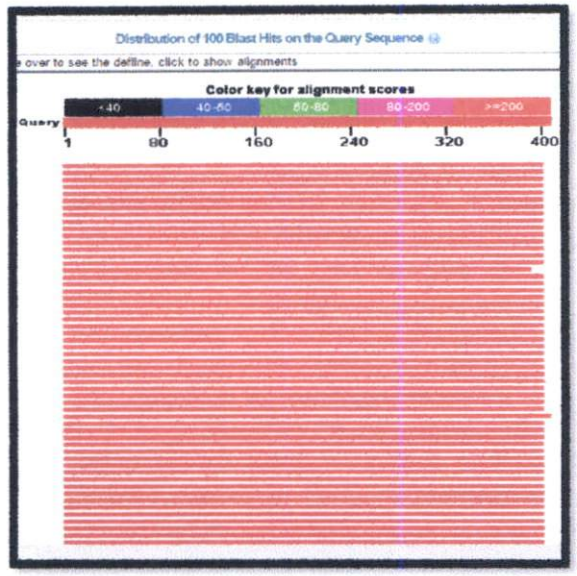


Figure 9: BLAST analysis of the SPLCV sequence

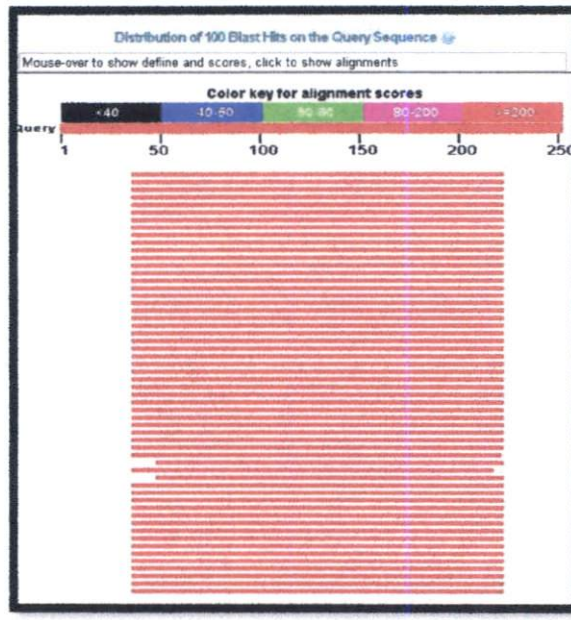


Figure 10: BLAST analysis of the SPVG sequence

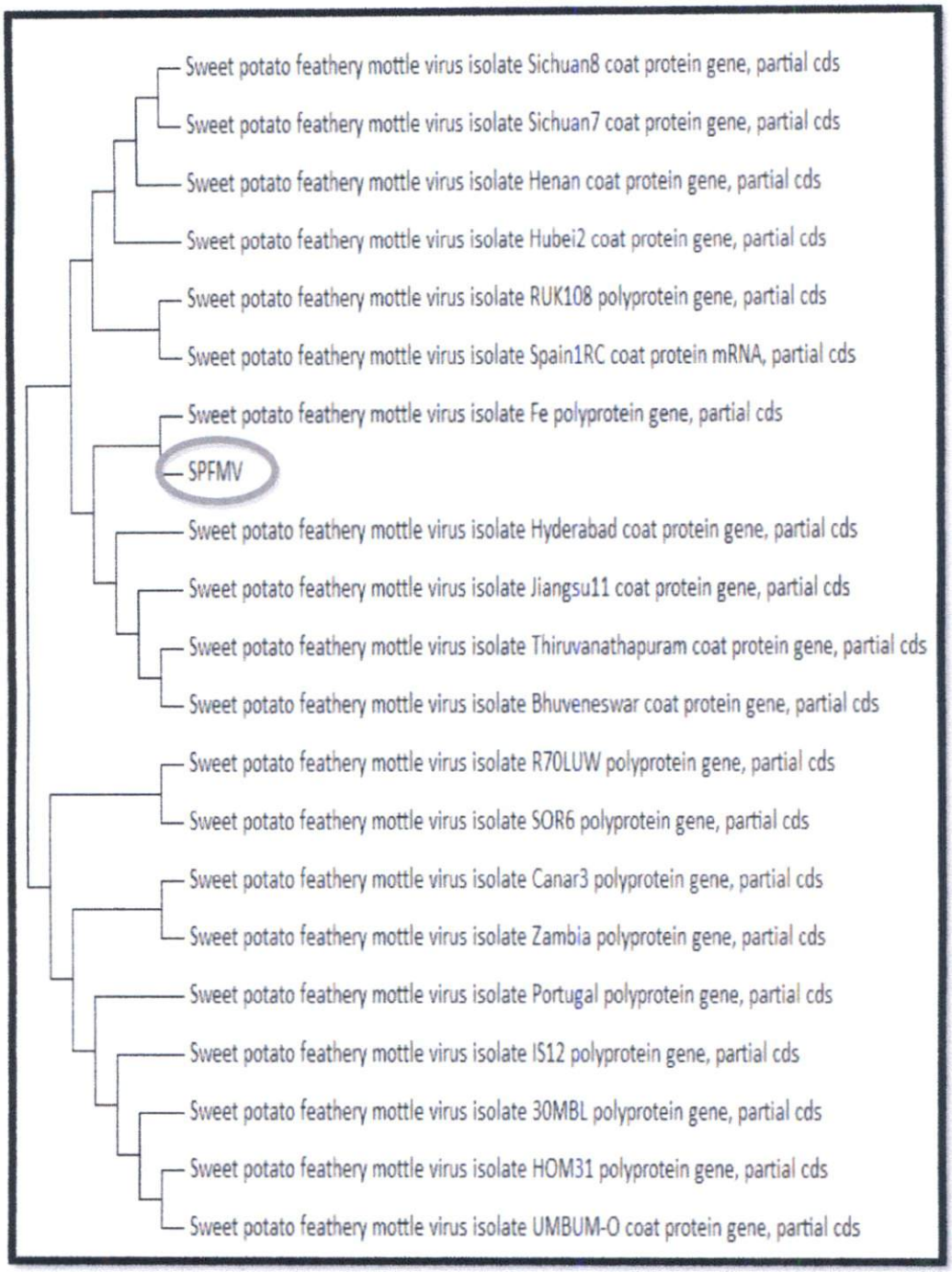


Figure 11: Phylogenetic tree construction of SPFMV sequence

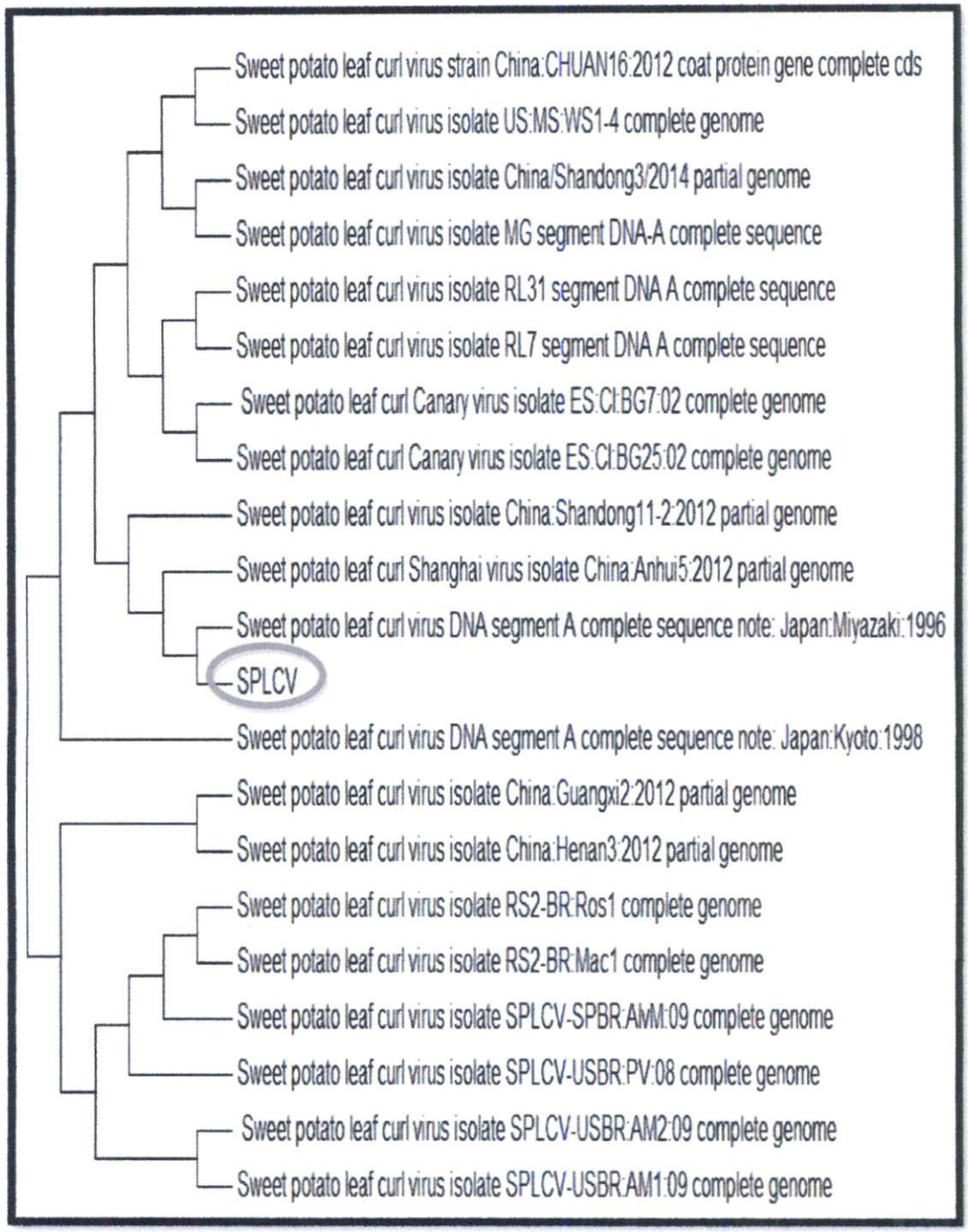


Figure 12: Phylogenetic tree construction of SPLCV sequence

Figure 13: Phylogenetic tree construction of SPVG sequence

DISCUSSION

5. DISCUSSION

Sweet potato (*Ipomoea batatas* (L.) Lam) is a dicotyledonous perennial plant belonging to the *Convolvulaceae*. *I. batatas* is the only food crop out of the approximately 500 species in this family (Watson and Dallwitz, 1991; 1994; Onwueme and Charles, 1994). Sweet potato is an important crop for food security (Gibson *et al.*, 2009). It is mainly grown in developing countries, which accounts for over 95% of world output. In India, sweet potato is cultivated mostly in Odissha, West Bengal, Bihar and Uttar Pradesh.

Sweet potato production is greatly constrained, particularly by viral diseases that cause yield reduction of over 50% (Gibson *et al.*, 1998; Mukasa *et al.*, 2006). Vegetative propagation should result in the build up of viruses from generation to generation (Okpul *et al.*, 2011). Sweet potato viruses are mainly spread through healthy looking vines, which farmers collect from the previous crop for the next cropping cycle. Thus singly infected vines can act as source of inoculums and through vector transmissions lead to mixed infections of different viruses (Rukarwa, *et al.*, 2010).

More than 30 viral diseases of sweet potato have been reported in different parts of the world (Kashif *et al.*, 2012). The most important and devastating viral disease affecting sweet potatoes worldwide is Sweet potato virus disease (SPVD). Sweet potato virus disease can reduce yields of infected plants by upto 80 per cent (Mukiibi, 1977; Hahn, 1979). Common viral diseases in sweet potato are caused by *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato yellow dwarf virus* (SPYDV) and *Sweet potato leaf curl Georgia virus* (SPLCGV). Multiple virus infections in sweet potato are a common phenomenon (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). SPVD is caused by synergetic interaction between a *Potyvirus*, SPFMV and a *Crinivirus*, SPCSV. SPMMV has occurred most frequently in mixed infections

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

The common viral diseases seen are SPFMV, SPMMV, *Sweet potato latent virus* (SPLV), *Sweet potato chlorotic fleck virus* (SPCFV) and *Sweet potato leaf curl virus* (SPLCV). SPFMV was first described in the United States about 60 years ago (Tairo *et al.*, 2005). It is the most important and widespread virus among the viruses detected in sweet potato (Moyer and Salazar, 1989). *Sweet potato feathery mottle virus* was detected in different samples in India (Jeeva *et al.*, 2004). Makeshkumar *et al.* (2007) observed some of the sweet potato lines with leaf curl symptoms. It is the first report of occurrence of SPLCV in India. In order to improve sweet potato production and to ensure quality sweet potato planting material, effective diagnostic method is a pre-requisite. Development of a breeding program first requires the correct identification of infecting viruses. This research programme envisages the identification and characterisation of viruses and their strains infecting sweet potato.

Viruses are one of the most important pathogens of sweet potato, with some infections resulting in severe yield reduction and plant death. Mixed infections of viruses are major constrain to the sweet potato production. There are 5 main viruses reported infecting sweet potato in India. *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato leaf curl virus* (SPLCV), *Sweet potato latent virus* (SPLV), *Sweet potato chlorotic fleck virus* (SPCFV). SPFMV, SPMMV and SPLCV are well characterized viruses. Electron microscopy studies revealed that the SPFMV is a potyvirus with an average length of 748 nm. The virus was purified from SPFMV infected sweet potato leaves. The antiserum was produced and tested using Ouchterlony agar double-diffusion test. *Sweet potato feathery mottle virus* was detected in different samples using direct antigen coating-ELISA and nitrocellulose membrane-ELISA (Jeeva *et al.*, 2004).

Babu *et al.* (2011) carried out reverse transcription polymerase chain reaction 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. Makeshkumar *et al.* (2007) observed some of the sweet potato lines with leaf curl symptoms. Total DNA was isolated from the infected plants and subjected to PCR using gemini group specific primer which has yielded positive amplification of 530 bp. Analysis of the sequence of PCR products showed close relationship with published SPLCV sequences. It is the first report of occurrence of SPLCV in India. Prasanth and Hegde (2008) collected cuttings from symptomatic sweet potato plants from Kerala, Odissha and Andhra Pradesh and maintained in insect-proof glass house. Total nucleic acids isolated from collected sweet potato samples were used for PCR and (RT)-PCR with gemini virus group specific primer and potyvirus specific primer. The expected 530 bp and 1.3 kb fragments were generated from the gemini virus and potyvirus primer sets, respectively. To further identify the viruses, nested primers specific for the coat protein gene of SPFMV and SPLCV were designed. Phylogenetic analysis with MEGA software program showed the highest sequence similarity with SPLCGV. But these studies were based on the identification of single virus only. The synergetic interactions of two or more viruses infecting sweet potato have not studied yet in India, although the devastating effects of such virus combinations were given keen attention all over the world. Thus it is important to study the presence of more than one virus causing mixed infections in sweet potato.

Since the 1970s, serological methods like ELISA have been used widely and successfully for detection of plant viral diseases (Clark and Adams, 1977; Flegg and Clark, 1979).ELISA techniques include NCM-ELISA and DIBA. But it has major limitations such as its low sensitivity during periods of low virus titre.

Moreover serological diagnosis of potyviruses is often impressive, because of frequent serological cross-reaction between species and biological indexing is very cumbersome (Brunt, 1992). Out of 37 samples, subjected for ELISA gave positive for SPFMV infection; while there is no single sample showed positive result for SPMMV. In DIBA, 7 samples were positive for SPFMV but all samples were negative for SPMMV. In this study, the viruses SPFMV, SPMMV, SPLCV, SPVG, SPCFV, SPVC and SPV2 were screened for their presence in the serological and nucleic acid based methods of virus detection. Initial screening using ELISA was used to detect SPFMV and SPMMV. Followed by ELISA screening, samples were subjected to PCR based virus detection using group specific and virus specific primers. Reverse transcription (RT) and PCR is used in plant virus detection. The PCR based methods are fast, highly sensitive and useful for accurate detection, quantification and characterization of plant pathogens. Degenerate primers are used for recognizing conserved regions of viral genomes. According to the ELISA and DIBA results, 32 samples were selected as the representative sample set. Among 8 pairs of potyvirus specific primers used 27 samples gave amplicon of 327 bp for MJ1/MJ2 primers while no amplification for CN48/oligo-dT (1500 bp) and Hrp5/oligo-dT (1500 bp) revealed the difficulty in amplifying large regions in potyvirus genome. Hence MJ1/MJ2 can be used as an universal primer for potyvirus detection. Out of 32 samples, 29 samples showed SPFMV infection in PCR based detection. The major symptoms commonly exhibited by these samples are pink colour ring spots, feathering, veinal chlorosis, faint-to-distinct chlorotic spots with or without purple margins, puckering. Samples without symptoms were also identified being infected with SPFMV. SPFMV was detected in large number of plants that were infected with other viruses.

In this study molecular characterization of samples is done by PCR with potyvirus specific primers MJ1/MJ2 and SPFMV specific primers SPFMV1/SPFMV2, amplifying the WCIEN and QMKAA motif of CP and partial

CP giving an amplicon of 327 bp and 411 bp respectively was found to be a robust of detecting SPFMV infections. Degenerate primers to conserved regions in the viral genomes are primarily used in the identification of potyviruses. They also enable partial genomic sequencing for taxonomic purposes. The group specific PCR and subsequent sequence analysis of the amplified region has been used for rapid detection and identification of potyvirus and is appeared to be the most suitable method for identification of viruses which are difficult to purify and/or occurring in mixed infections (Colinet *et al.*, 1998). Marie Jeanne et al. (2000) proved RT-PCR with MJ1/MJ2 primers useful for the detection and identification of the potyviruses infection. Other potyvirus group specific primers like Atropa Nad2.1a/ Atropa Nad2.2b amplifying NAD region, NIb2F/NIb3R amplifying NIb region, CN48/oligo-dT amplifying WCIEN motif of CP, Pot1/Pot2 amplifying NIb and CP region, Hrp-5/oligo-dT amplifying 3'end, MJ1/M4T amplifying C-terminal region of CP and 3' UTR and Pot1/Hrp-5 amplifying 3'end gave amplicons of 188 bp/800 bp, 350 bp, 700 bp, 1300 bp, 1500 bp, 719 bp and 700 bp/1300 bp respectively.

SPLCV is the another major virus detected in the samples with curling symptoms more severely infected with SPLCV, which was detected by LCV1/LCV2 virus specific primers coding for the partial CP giving an amplicon size of 446 bp. There were 16 samples showing SPLCV infections and 15 of them showed mixed infections with SPFMV. According to Revill *et al.* (2005a) some of the viruses occur as latent infections, and all can be formed in various combinations; this means that it is difficult to ascribe symptoms to infection by individual viruses. Even though symptoms similar to SPMMV like yellow netting was observed in samples S1322 and S570, none of them showed positive results in ELISA and PCR. As these samples were positive for SPFMV ELISA and showed symptoms of SPMMV, PCR detection confirmed the virus as SPFMV. None of the samples showed amplification in PCR for SPV2, SPVC and SPCFV. There was no amplification for sample S270 in PCR for SPVG with primers SPG-F1/SPFCG2-R2, but it showed high sequence

similarity with SPVG in BLAST analysis when 1300 bp size amplicon of Pot1/Hrp-5 group specific primers was sequenced. Hence SPVG, a lenient of SPFMV was confirmed. The sample S270 was mixedly infected with SPFMV, SPLCV and SPVG. The samples S1294, S684 and S270 which showed amplicons for SPFMV, SPLCV and SPVG respectively were cloned for the confirmation of the respective viruses.

The 304 nt SPFMV sequence obtained in this study showed maximum similarity of 96% to *Sweet potato feathery mottle virus isolate Fe polyprotein gene, partial cds* (Accession EU021070). The 251 nt SPVG sequence obtained in this study showed maximum similarity of 90% to *Sweet potato virus G isolate IS103, complete genome* (Accesssion KM014815). The 418 nt SPLCV sequence obtained in this study showed maximum similarity of 96% to *Sweet potato leaf curl virus DNA A, complete sequence* (Accession AF104036) and *Sweet potato leaf curl isolate CTCRI TVM M1, complete genome* (Accession KM050768). According to Fanquet *et al* (2005) potyvirus isolates with 85% sequence identity or more over the whole genome are usually considered to be from the same species.

SUMMARY

6. SUMMARY

The study entitled "identification and characterization of viruses in sweet potato (*Ipomoea batatas* (L) Lam.) was carried out at the division of crop protection, Central Tuber Crops Research Institute, Sreekariyam, Trivandrum during 2014-2015. The objective of the study was to diagnose, clone and characterize viruses implicated in mixed infections of sweet potato. The important findings of the above studies are summarized in this chapter.

Sweet potato samples with various virus infection symptoms were collected from the germplasm repository of CTCRI, Trivandrum and field samples from Bhubaneswar. Serological screening was done for SPFMV and SPMMV. A representative sample set of 32 sweet potato leaf samples were made based on the result of ELISA, DIBA and common symptoms such as feathering, puckering, pink colour ring spots, veinal chlorosis, faint -to-distinct chlorotic spots with or without purple margins, stunting, upward curling or rolling of leaves. These samples were screened mainly for *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato leaf curl virus* (SPLCV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato virus G* (SPVG), *Sweet potato virus C* (SPVC), *Sweet potato virus 2* (SPV2) using both genus and virus specific primers. Out of 32, 29 samples showed SPFMV infection and 16 samples for SPLCV in PCR with virus specific primers. While mixed infection by SPFMV and SPLCV was found in 15 samples. One sample was infected with SPVG along with SPFMV and SPLCV. There was no infection of SPMMV as the serological and PCR screening gave negative results. SPVC, SPV2 and SPCSV screening through PCR gave negative results for all samples.

This study found out that SPFMV and SPLCV to be the most common virus infecting sweet potato in India, the former being ubiquitous in sweet potato

everywhere. The samples characterized suggests feathery symptoms, puckering, pink colour ring spots, veinal chlorosis, faint-to-distinct chlorotic spots with or without purple margins to be the common symptoms associated with SPFMV. The samples with these prominent symptoms and samples showing no such symptoms were identified with SPFMV. Stunting, upward curling or rolling of leaves were the major symptoms shown by some sample plants. When these samples were subjected to characterization they were confirmed with the presence of SPLCV. Yellow netting shown by two samples seemed to be infected with SPMMV, but molecular characterization proved these samples are infected with SPFMV. Another major finding of this study is the presence of SPVG, a lenient of SPFMV which was not common in India. The symptoms shown by this sample were same as the symptoms shown by SPFMV infected samples. This showed the RNA viruses are highly prone to variations. As earlier studies were done for identification of single viruses only, this study envisaged the identification of mixed infections with multiple viruses. It was clear that SPFMV has a synergetic interaction with SPLCV as the number of samples co-infected with both was 15. These samples with mixed infections showed the major symptoms of both SPFMV and SPLCV like feathering, pink colour ring spots, upward curling of leaves. When they were subjected to PCR by virus specific primers of SPFMV and SPLCV, amplicons size of 411 bp and 446 bp were obtained respectively.

PCR based diagnostics carried out using potyvirus specific primer MJ1/MJ2 and SPFMV1 /SPFMV2 amplifying the WCIEN and QMKAA motif of CP and partial CP giving an amplicon of 327 bp and 411 bp respectively was found to be a robust of detecting SPFMV infections in India. Rather than the virus specific primers, the group specific primers Pot1/Hrp5 lead to the detection of SPVG. The virus specific primers LCV1/LCV2 coding for the partial CP giving an amplicon of 446 bp were efficient to detect SPLCV.

After identification, one sample each for SPFMV, SPLCV and the only sample positive for SPVG were cloned and sequenced. The sequence data was analyzed through BLAST and sequence similarity was studied. The 304 nt SPFMV sequence obtained in the study showed maximum similarity of 96% to *Sweet potato feathery mottle virus isolate Fe polyprotein gene, partial cds* (Accession EU021070). The 251 nt SPVG sequence obtained showed maximum similarity of 90% to *Sweet potato virus G isolate IS103, complete genome* (Accession KM014815). While the 418 nt SPLCV sequence obtained showed maximum similarity of 96% to *Sweet potato leaf curl virus DNA A, complete sequence* (Accession AF104036) and Sweet *potato leaf curl isolate CTCRI TVM M1, complete genome* (Accession KM 050768). The phylogenetic tree was constructed with similar sequences. Phylogenetic analysis clearly revealed that the sequences obtained in this study belongs to SPFMV for the sample S1294, SPLCV for the sample S1294, SPLCV for the sample S684 and SPVG for the sample S270 as they grouped along with their respective virus sequences used for comparison analysis.

Out of major sweet potato affecting viruses worldwide, *Sweet potato feathery mottle virus* belonging to the *Potyvirus* and *Sweet potato leaf curl virus* belonging to the *Begomovirus* are widely found regionally. Sample studies showed that there is a high rate of mixed infections caused by SPFMV and SPLCV in sweet potato. And the chances of variations in RNA viruses are leading to the new viral infection like *Sweet potato virus G*.

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APPENDIX

8. APPENDIX

ELISA Coating Buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.20 g

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

ELISA Sample Extraction Buffer

PBS-T (1X PBS + 0.5ml Tween 20/L) + 2% PVP

ELISA Conjugate Buffer

PBS-T (1X PBS + 0.5ml Tween 20/L) + 2% PVP + 0.2% egg albumin

ELISA Substrate Buffer

Diethanolamine	9.7 ml
Distilled Water	80 ml
NaN_3	0.2 g

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

ELISA Blocking Buffer

PBS-T (1X PBS + 0.5ml Tween 20/L) + 2% dried skimmed milk

CTAB RNA Extraction Buffer

Tris- HCl (pH 8.0)	100 m*M*
EDTA	25 m*M*
NaCl	2 *M*
CTAB	2 %

β-mercaptoethanol	2 % (v/v)	freshly added prior to RNA
PVP	2 % (w/v)	extraction

Prepared in DEPC treated water.

TAE Buffer (50X)

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

CTAB DNA Extraction Buffer

Tris- HCl (pH 8.0)	100 m*M*	
EDTA	20 m*M*	
NaCl	1.4 *M*	
CTAB	2 %	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA
PVP	2 % (w/v)	extraction

Luria Agar Medium

35.0 grams of LA (HiMedia) in 1000 ml distilled water

T Solution

Mix 250 µl T- Solution (A) and 250 µl T- Solution (B)

LB Medium

20 g of LB (HiMedia) in 1000 ml distilled water

LB Ampicillin X gal/ IPTG Agar Plates

LB + Ampicillin 0.1mM +X-gal 40 µg/ml + IPTG 0.1mM

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF VIRUSES IN SWEET POTATO

[*Ipomoea batatas* (L.) Lam.]

by

JAYALEKSHMI V. S.

(2010-09-102)

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9. ABSTRACT

The study entitled "identification and characterization of viruses in sweet potato (*Ipomoea batatas* (L) Lam.) was carried out at the division of crop protection, Central Tuber Crops Research Institute, Sreekariyam, Trivandrum during 2014-2015. The objective of the study was to diagnose, clone and characterize viruses implicated in mixed infections of sweet potato.

Sweet potato samples with various virus infection symptoms were collected from the germplasm repository of CTCRI, Trivandrum and field samples from Bhubaneswar. Samples were screened mainly for *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato leaf curl virus* (SPLCV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato virus G* (SPVG), *Sweet potato virus C* (SPVC), *Sweet potato virus 2* (SPV2) using both genus and virus specific primers. Out of 32, 29 samples showed SPFMV infection in PCR with virus specific primers. While mixed infection by SPFMV and SPLCV was found in 15 samples. One sample was infected with SPVG along with SPFMV and SPLCV. SPMMV, SPVC, SPV2 and SPCSV screening through PCR gave negative results for all samples.

PCR by virus specific primers of SPFMV and SPLCV amplifying the partial CP gave amplicons size of 411 bp and 446 bp respectively. Rather than the virus specific primers, the group specific primers Pot1/Hrp5 gave an amplicon of 1300 bp lead to the detection of SPVG. After identification, one sample each for SPFMV, SPLCV and the only sample positive for SPVG were cloned and sequenced. The sequence data was analyzed through BLAST and sequence similarity was studied. The 304 nt SPFMV sequence obtained in the study showed maximum similarity of 96% to *Sweet potato feathery mottle virus isolate Fe polyprotein gene, partial cds* (Accession EU021070). The 251 nt SPVG sequence obtained showed maximum similarity of 90% to *Sweet potato virus G isolate IS103, complete genome*

(Accession KM014815). While the 418 nt SPLCV sequence obtained showed maximum similarity of 96% to *Sweet potato leaf curl virus DNA A, complete sequence* (Accession AF104036) and *Sweet potato leaf curl isolate CTCRI TVM M1, complete genome* (Accession KM 050768). The phylogenetic tree was constructed with similar sequences. Phylogenetic analysis clearly revealed that the sequences obtained in this study belongs to SPFMV for the sample S1294, SPLCV for the sample S684 and SPVG for the sample S270 as they grouped along with their respective virus sequences used for comparison analysis.

Since the diagnosis of virus infections based on symptoms is unreliable due to complicated mixed infections in sweet potato with multiple viruses and isolates, it is necessary to develop region wise sensitive diagnostic tests to confront this issue. As a prerequisite to this, virus detection and identification has to be carried out in sweet potato to determine the viruses geographically.