# DIVERSITY OF BEGOMOVIRUSES INFECTING MAJOR VEGETABLE CROPS

*by*

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**2019**

# DECLARATION

I, hereby declare that this thesis entitled **"Diversity of begomoviruses infecting major vegetable crops"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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# CERTIFICATE

Certified that this thesis entitled **"Diversity of begomoviruses infecting major vegetable crops"** is a record of research work done independently by Ms. Bincy S. Basheer under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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## LIST OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| % | Per cent |
| µl | Microlitre |
| °C | Degree Celsius |
| bp | base pair |
| O.D | Optical density |
| DNA | Deoxyribo nucleic acid |
| *et al.* | And other co-workers |
| Fig. | Figure |
| g | Gram |
| h | Hour |
| KAU | Kerala Agricultural University |
| kb | Kilobase pair |
| kg | Kilogram |
| L | Litre |
| mg | Milligram |
| min. | Minute |
| ml | Millilitre |
| M | Molar |
| mM | Millimolar |
| ng | Nanogram |
| N | Normality |
| D.I. | Disease incidence |
| V.I. | Vulnerability index |
| PCR | Polymerase chain reaction |
| RCA | Rolling circle amplification |
| CP | Coat protein |
| ELISA | Enzyme linked immunosorbent assay |
| TAS-ELISA | Triple antibody sandwich-Enzyme linked immunosorbent assay |
| DAS-ELISA | Double antibody sandwich-Enzyme linked |

| | |
|---|---|
| | immunosorbent assay |
| Ab | Antibody |
| RFA | Restriction Fragment Analysis |
| rpm | Rotations per minute |
| sec | Second |
| Sl. | Serial |
| temp. | Temperature |
| V | Voltage |
| *viz.* | Namely |
| M | Molarity |
| DAG | Days after grafting |
| DAI | Days after inoculation |
| nm | nanometre |
| AAP | Acquisition access period |
| IAP | Inoculation access period |
| PVP | Poly vinyl pyrolydone |
| PAb | Polyclonal antibody |
| MAb | Monoclonal antibody |
| DIBA | Dot immunobinding assay |
| NCM | Nitrocellulose membrane |
| NBT | Nitroblue tetrazolium |
| BCIP | Bromochloroindolyle phosphate |
| SDM | Spray dried milk |
| bp | Base pairs |
| CTAB | Cetyl trimethyl ammonium bromide |
| TAE | Tris-acetate-EDTA |
| TE | Tris-EDTA |
| EDTA | Ethylene diamine tetra acetic acid |
| EtBr | Ethydium bromide |
| TBS | Tris buffer saline |
| PBS | Phosphate buffer saline |

| | |
|---|---|
| PBST | Phosphate buffer saline-Tween |
| BYVMV | *Bhendi yellow vein mosaic virus* |
| ToLCV | *Tomato leaf curl virus* |
| PYVMV | *Pumpkin yellow vein mosaic virus* |
| BYVMD | *Bhendi yellow vein mosaic disease* |
| ToLCD | *Tomato leaf curl disease* |
| PYVMD | *Pumpkin yellow vein mosaic disease* |
| SLCCNV | *Squash leaf curl China virus* |
| ACMV | *African cassava mosaic virus* |
| SLCMV | *Sri Lankan cassava mosaic virus* |
| ToLCNDV | *Tomato leaf curl New Delhi virus* |
| ToLCBV | *Tomato leaf curl Bangalore virus* |
| ToLCKeV | *Tomato leaf curl Kerala virus* |
| ToLCKV | *Tomato leaf curl Karnataka virus* |
| ToLCGV | *Tomato leaf curl Gujarat virus* |
| SLCV | *Squash leaf curl virus* |
| TYLCV | *Tomato yellow leaf curl virus* |
| BgYMV | *Bittergourd yellow mosaic virus* |

# *Introduction*

# 1. INTRODUCTION

The worldwide expansion of agriculture has resulted in the emergence and spread of numerous plant diseases. Plant viruses are reported to be the causal agents of many economically important plant diseases all over the world. In India majority of the vegetable crops are infected with plant viruses. Among them, bhendi yellow vein mosaic disease, tomato leaf curl disease and pumpkin yellow vein mosaic disease are caused by plant viruses that belong to the family *Geminiviridae.*

Geminiviruses are small plant-infecting arthropod borne viruses with single-stranded DNA (ssDNA) genomes of size approximately 2.6 kb that are encapsidated in twinned (geminate) quasi-isometric particles (Stanley *et al.*, 2005). Geminiviruses make up a large, diverse family of plant viruses that infect a broad variety of food and fiber crops; and cause significant crop losses worldwide. Begomoviruses, the largest of all the nine genera of Geminivirus, are transmitted by the whitefly (*Bemisia tabaci*) and infect mainly dicotyledonous plants. Begomoviruses had emerged everywhere in the world where environmental conditions supported large populations of *B. tabaci* and became a major threat for the production of food and fiber crops (Varma and Malathi, 2003). Begomoviruses could be bipartite (having two DNA molecules, DNA-A and DNA-B) or monopartite (having a single DNA molecule, resembling DNA-A) (Fondong, 2013). The majority of begomoviruses have a genome comprising two similar sized DNA components (DNA-A and DNA-B).

Okra (*Abelmoschus esculentus* (L.) Moench), commonly known as bhindi in India, is an important vegetable grown and consumed in the Indian subcontinent. Okra originated in tropical Africa (Purseglove, 1987), also called Lady's finger belongs to the family Malvaceae, is an important vegetable throughout the tropical and subtropical regions of the world. It is a very common vegetable in India and popular among all classes of people.Swanson and Harrison (1993) and Brunt *et al.*

(1996) reported that at least 19 different plant viruses were found in okra. Viral diseases of okra are the major constraints to their cultivation and the majority of the viral diseases in okra are caused by begomoviruses and various alpha- and beta-satellites (Rishishwar *et al.,* 2015).

Among the viral diseases observed, yellow vein mosaic disease (YVMD) transmitted by white fly (*Bemisia tabaci* Gen.) is the most important and devastating viral disease observed in Indian subcontinent (Prakasha *et al.,* 2010).

The first record of the disease was from Bombay in India (Kulkarni, 1924) and subsequently named as "yellow vein mosaic of okra" (Uppal *et al.*, 1940). This is the earliest report of *Bhendi yellow vein mosaic virus* (BYVMV), implying that BYVMV might have originated in India. BYVMV had been shown to be a Geminivirus based on its morphology and its serological relationship with *African cassava mosaic virus* (ACMV) (Harrison *et al.,* 1991). Okra plants infected by BYVMV expressed persistent symptoms of vein clearing followed by yellowing. Leaves and fruits were reduced in size leading to a significant decrease in the production of the vegetable. Infection of cent per cent plants in a field was very common and the yield loss ranged from 50 to 94 per cent depending on the stage of the crop growth at which the infection occurs (Sastry and Singh, 1973). Up to 96 per cent loss in yield had been reported due to BYVMV infection in okra (Pun and Doraiswamy, 1999).

Tomato (*Lycopersicon esculentum* L.) is another most widely grown vegetable crop due to its high nutritive value, taste and versatile use (Solieman *et al.,* 2013). In 2015, global production of tomato was 16.38 mt with an area of 0.0643 mha (NHB, 2015). Tomato is susceptible to more than 200 diseases caused by fungi, bacteria and viruses. However, 40 viral diseases were reported in tomato. Among these viruses, *Tomato leaf curl virus* (ToLCV) is a major Geminivirus which causes serious loss to tomato production in tropical and subtropical regions of the world.

Tomato leaf curl disease (ToLCD) was first reported in northern India by Vasudeva and Samraj (1948). In the 1950s, ToLCD was reported in central India (Varma, 1959), and a few years later it was reported in the main tomato growing region of Karnataka state, South India (Govindu, 1964; Sastry and Singh, 1973). Symptoms of ToLCD included leaf curling, vein clearing and stunting, and if infection occurs at the seedling stage, plants became sterile (Saikia and Muniyappa, 1989). ToLCV causing leaf curl disease in tomato led a heavy toll on the crop and caused a yield loss up to cent per cent (Saikia and Muniyappa, 1986; Kalloo, 1996).

ToLCD is caused by a range of circular ssDNA virus species in the genus Begomovirus (Polston and Anderson, 1997; Fauquet and Stanley, 2003). Old World tomato viruses, *Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus*-[AU] lack a DNA-B component, requiring only their DNA-A component to systemically and symptomatically infect plants (Dry *et al.,* 1993; Kheyr-Pour *et al.,* 1991; Navot *et al.,* 1991). Diversity in *Tomato leaf curl virus* (ToLCV) in southern India has been apparent since the early 1980s (Reddy *et al.*, 1981). The association of these satellite molecules was found to enhance the symptom severity (Kumari *et al.,* 2010). Beta-satellites have been reported along with bipartite begomoviruses like *Tomato leaf curl New Delhi virus* (ToLCNDV) (Sivalingam *et al.,* 2010; Jyothsna *et al.,* 2013).

Cucurbits are economically important group of vegetable crops cultivated throughout India and the Begomovirus-associated diseases in cucurbits are emerging rapidly in recent years. The Begomovirus diseases have been detected time to time in India on cucurbitaceous crops such as bitter gourd, cucumber, muskmelon, winter squash, bottle gourd, sponge gourd, ridge gourd, ivy gourd, pumpkin and watermelon by nucleic acids hybridisation tests or by PCR using primers of putative coat protein (CP) gene of ToLCNDV (Khan *et al.*, 2002; Sohrab *et al.*, 2006). Pumpkin (*Cucurbita moschata* Duch. Ex Poir) is an important vegetable grown extensively in

India throughout the year for its mature and immature fruits. The crop is infected with several virus diseases like pumpkin mosaic (PM), pumpkin yellow vein mosaic (PYVM), squash leaf curl mosaic, pumpkin enation mosaic and pumpkin mild mosaic (Ghosh and Mukhopadhyay, 1979). The cultivation of pumpkin has often suffered serious setback due to severe outbreak of mosaic diseases, particularly yellow vein mosaic and pumpkin mosaic (Latha, 1992).

PYVM was reported in northern India by Vasudeva and Lal in 1943. Incidence of PYVM and the associated yield losses were reported to be high during February to May, when the vector population is at its peak (Muniyappa *et al.*, 2003). A yield loss of up to 90 per cent had been recorded in pumpkin due to this disease (Singh *et al.*, 2009). In India PYVMV was reported to be caused by bipartite begomoviruses such as *Tomato leaf curl New Delhi virus-India* and *Squash leaf curl Palampur virus* (Jaiswal *et al.*, 2011) from North India (Maruthi *et al.*, 2007), *Squash leaf curl China virus-India* from South India (Muniyappa *et al.,* 2003; Singh *et al.,* 2007).

Viral diseases are very difficult to control due to the lack of efficient chemicals. Only practical solution of this problem is to develop tolerant / resistant varieties (Ali *et al.,* 2000). Hence epidemiological studies are relevant for outlining the management of viral diseases. In this context, the modes of transmission as well as genetic diversity are of important consideration. Recent studies of begomovirus populations from the Indian subcontinent revealed their evolutionary divergence as a new group of begomoviruses, distinct from other begomoviruses causing similar diseases in other geographical regions of the world (Lefeuvre *et al.,* 2007). Hence the present study was attempted to collect the begomoviruses infecting okra, tomato and pumpkin plants; to characterize the viruses immunologically and molecularly; and to study the phylogeny and genetic diversity among the viruses.

# *Review of Literature*

## 2. REVIEW OF LITERATURE

Among several fungal, bacterial and viral diseases; bhendi yellow vein mosaic disease (BYVMD), tomato leaf curl disease (ToLCD) and pumpkin yellow vein mosaic disease (PYVMD) caused by begomoviruses were reported to be a serious constraint in the production of the respective crops across India (Uppal *et al.*,1940; Maruthi *et al.*, 2007). BYVMD, ToLCD and PYVMD were caused by begomoviruses that belong to Geminiviridae family.

Geminiviruses have characteristically twinned or ''geminate'' particle morphology. The capsid had two joined, incomplete T = 1 icosahedral heads, with 110 molecules of the protein organized as 22 pentameric capsomers (Zhang *et al.*, 2001). Geminate particle consisted of twinned quasi-icosahedral virus particle (virion) of ~18 X 30 nm and a small circular single stranded DNA genome of ~2.9-5.2 kb. The family name is derived from the distinctive twinned virions (the Latin word gemini means twin) (Brown *et al.*, 2012). Based on genome structure, phylogenetic relationships, insect vector and host range, nine genera had been recognized *viz.*, Begomovirus, Mastrevirus, Curtovirus, Topocuvirus, Becurtovirus, Turncurtovirus, Eragrovirus, Grablovirus and Capulavirus (ICTV, 2017).

The genus Begomovirus (family Geminiviridae) is the largest genus of plant viruses with respect to number of species included. In fact, with 288 species currently recognized by the International Committee on Taxonomy of Viruses (ICTV) (http://www.ictvonline.org/virusTaxonomy.asp), it is the largest genus of all viral taxonomy (Brown *et al.*, 2015). Based on their genome organization, genetic diversity and geographical distribution, begomoviruses can be divided into two groups; Old World resembling Europe, Africa, Asia and Australia; and New World resembling the American continents (Nawaz-ul-Rehman and Fauquet, 2009).

Begomoviruses had either bipartite (DNA-A and DNA-B) or monopartite (equivalent of DNA-A) genomes, with A component encoding 5–7 proteins and B component encoding two proteins, each component being 2.5–2.8 kb in size (Fondong 2013). The majority of begomoviruses have a genome comprising two similar sized DNA components (DNA-A and DNA-B). The two components of begomoviruses have only a little sequence homology except for an intergenic region (IR), which includes the common region (CR) of approximately 200 nucleotides with typically more than 85% identity between DNA-A and DNA-B. The CR contains a highly conserved (among geminiviruses) hairpin structure with the characteristic nonanucleotide sequence (TAATATTAC) that marks the origin of virion-strand DNA replication (Hanley-Bowdoin *et al.*,1999), repeated sequences (collectively known as "iterons") required for the binding of the replication (Rep) protein (Arguello-Astorga *et al.,* 1994) and regulatory regions for bidirectional transcription. The CR thus functions in integrity maintenance of the divided genome and in ensuring the replication of both components initiated by the DNA-A-encoded Rep protein (Briddon *et al.,* 2010). The DNA-A component is capable of autonomous replication and can produce virus particles that encode proteins responsible for particle encapsidation (CP) (V1), viral replication (replication-associated protein (Rep) and replication enhancer protein (REn), regulation of gene expression (transcription activator protein, TrAP) and suppression of host defense mediated by gene silencing (C2). Modulation of symptom development and suppressor of gene silencing were attributed to the protein encoded by C4 gene of begomoviruses originating from the Old World. The New World begomoviruses lack an ORF termed V2 but it is found in begomoviruses originating from the Old World. The V2 is involved in movement and pathogenicity of the viruses. The ORF BV1 and BC1 located on DNA-B component encode two proteins involved in intracellular (nuclear shuttle protein- NSP) and intercellular virus movement (movement protein, MP) (Lazarowitz, 1992).

Some begomoviruses had only a single genomic component which resembles DNA-A, such as isolates of *Tomato yellow leaf curl virus* (TYLCV), *Tomato leaf curl virus* (TLCV), *Ageratum yellow vein virus* (AYVV) and *Cotton leaf curl virus* (CLCuV) (Kheyr-Pour *et al.,* 1991; Navot *et al.,* 1991; Dry *et al.,* 1993; Tan *et al.,*1995; Briddon *et al.,* 2000). In the past few decades, several small circular molecules, approximately half the size of begomoviruses, had also been reported to be present in plants infected with begomoviruses. These are known as alpha and beta satellites, depend on the cognate begomovirus for some of their basic functions. Alpha satellites encode a replication-associated protein, which resembles similar proteins of nanoviruses, a class of single-stranded DNA viruses having multipartite genome (Gronenborn, 2004; Idris *et al.,* 2011). Beta satellites, on the other hand encodes a single protein (βC1), which suppresses RNA-interference, a plant defence reaction against viruses (Saunders *et al.,* 2004; Saeed *et al*., 2005), hence contributing to the viral infectivity and virulence.

Recent studies of begomovirus populations from the Indian subcontinent revealed their evolutionary divergence as a new group of begomoviruses, distinct from other begomoviruses causing similar diseases in other geographical regions of the world (Lefeuvre *et al.,* 2007). High diversity among begomovirus species associated with mixed infections might be the major facilitator for the recombination and pseudorecombination events, leading to the frequent emergence of new begomoviruses (Jeske *et al.,* 2001; Schnippenkoetter *et al.,* 2001). The important source of evolution of Begomoviruses had been suggested either through mutation and/or recombination (Harrison *et al.,* 2002; Kirthi *et al.*, 2002). Maruthi *et al.* (2007) recommended that recombination might arise due to mixed infections of different Begomoviruses in a common host plant. Tiwari *et al.* (2012) speculated that existence of genetic diversity among Begomoviruses might be due to the occurrence of variants of *Bemisia tabaci.*

## 2.1 COLLECTION OF BEGOMOVIRUS INFECTED OKRA, TOMATO AND PUMPKIN

The survey conducted by Venkataravanappa (2008) on Geminivirus associated with okra in India revealed that the occurrence of yellow vein mosaic disease incidence ranged from 23.0–67.67 per cent in Karnataka, 45.89–56.78 per cent in Andhra Pradesh, 23–75.64 per cent in Tamil Nadu, 42.45–75.64 per cent in Kerala. The survey conducted in various okra fields of Aligarh (India) for the report of incidence of bhendi yellow vein mosaic disease (BYVMD) in 2012 kharif and rabi seasons also revealed that BYVMD incidence ranged from 1.5 to 19.5 per cent in rabi and 23.5 to 36.8 per cent in kharif season (Sheikh *et al.,* 2013 ). Venkataravanappa *et al.* (2015) collected 173 okra leaf samples from the plants exhibiting yellow mosaic, vein thickening, petiole bending, complete yellowing, upward curling with yellowing and stunted growth symptoms from different geographical locations representing India for studying the genetic diversity of begomoviruses infecting okra. Senevirathna *et al.* (2016) reported the highest incidence of BYVMD (90 %) from Sri Lanka with the characteristic symptoms of vein clearing of leaves and bleaching of fruits.

Saikia and Muniyappa (1986) and Kalloo (1996) reported that ToLCD caused yield loss up to cent per cent. The disease incidence recorded in Pantnagar, Uttarakhand was 35 per cent and 69 per cent in winter and summer respectively (Saklani and Mathai, 1977). Anandhan *et al.* (2011) observed the leaf curl disease of tomato in Haldwani region of Uttarakhand, India during 2004–2007 with an average disease incidence for five years as 49.8 and 73.7 per cent in October and February respectively with a higher disease incidence of 83 per cent in October 2004.

Jyothsna *et al.* (2013) carried out a survey between 2003 and 2010 to determine the distribution and genetic diversity of begomoviruses that infect solanaceous and cucurbitaceous plants (Tomato, potato, and cucurbit). 137 samples

were collected with the symptoms such as leaf puckering, leaf distortion, yellow mosaic, vein clearing, and leaf curl; and analyzed the presence of begomoviruses in the infected samples.

The incidence of PYVMD and the associated yield loss were high in cucurbits during February to May, when the vector population was its peak (Saikia and Muniyappa, 1989).

## 2.2 SYMPTOMATOLOGY

Yellow vein mosaic disease (YVMD) of okra was characterized by vein clearing, chlorosis, swelling of veins coupled with slight downward curling of leaf margins, twisting of petioles and retardation of growth (Capoor and Varma, 1950). Pun and Doraiswamy in 1999 reported that okra plants infected by BYVMV expressed persistent symptoms of vein clearing which later turned into complete chlorosis. A reduction in leaf and fruit size was reported by Baghat *et al.* (2001). Jose and Usha (2003) reported that the symptoms of the disease include yellow mosaic, vein thickening, vein twisting, petiole bending, complete yellowing, yellowing with upward curling and stunted plant growth. Under field conditions, disease symptoms start with the yellowing of the veins on the leaves.

According to Ali *et al.* (2000), depending on the intensity and distribution of infection on the plant, four distinct types of symptoms were noticed. In case of early infection at early stage of crop, symptoms expressed include yellowing of leaves which later turned brown and dried up and the infected plants hardly produced flowers and fruits; and ultimately died prematurely. While infection at later stage of growth caused symptoms on upper leaves and flowering parts with production of a few fruits that were yellow and hard at picking stage. In the case of mild infection, the uppermost leaves showed slight yellowing and fruits became light yellow

although they were mostly well developed or the plants continued to grow in a healthy state and yielded as good as symptomless plants.

Muniyappa *et al.* (2003) reported that symptoms on pumpkin plant as a result of yellow vein mosaic disease infection appeared as faint vein clearing in tertiary veins of younger leaves, which extend gradually to secondary and primary veins with prominent vein yellowing and coalescing to yellow mosaic. Maruthi *et al.* (2007) and Singh *et al.* (2009) reported that vein yellowing on older leaves caused early senescence leading to retardation of the plant growth. Apart from the typical vein clearing, other symptoms included reduction in leaf size, premature senescence of flowers and production of fruits unfit for marketing. Namratha *et al.* (2010) also reported similar symptoms that included vein yellowing, mosaic and crumpling of leaves, and production of distorted small fruits.

The tomato leaf curl disease in India was first reported by Vasudeva and Samraj (1948). The symptoms of ToLCD were mosaic, interveinal yellowing, vein clearing, and crinkling and puckering of the leaves accompanied by inward rolling of the leaf margins. The older leaves become leathery and brittle. The disease also induced severe stunting, bushy growth, and partial or complete sterility depending on the stage at which the infection had taken place. Infected plants produced few or no fruit (Vasudeva and Samraj, 1948; Nariani and Vasudeva, 1963; Muniyappa *et al.*, 1991). Reddy *et al.* (1981) observed various symptoms such as severe leaf curl with thickening of veins, enation, screw pattern of leaf arrangement, vein purpling and exclusively downward curling of the leaves. Pandey *et al.* (2010) and Kumari *et al.* (2011) reported yellowing and purplish discoloration of leaves in ToLCD.

## 2.3 TRANSMISSION STUDIES

### 2.3.1 Insect transmission

Varma (1955) was the first to report successful transmission of Begomovirus by *Bemisia tabaci*, which was later confirmed by Capoor and Ahmad (1975) and Jayashree *et al.* (1999) through transmission of PYVMV in pumpkin plants. Begomoviruses were transmitted exclusively by the sweetpotato whitefly *B. tabaci* in a persistent-circulative manner, although one report suggested that the castor bean whitefly *Trialeurodes ricini* can also transmit *Tomato yellow leaf curl virus* (TYLCV) (Idriss *et al.*, 1997). Weerasinghe (2010) reported that ToLCNDV causing leaf curl and severe mosaic disease in bittergoourd was transmitted only through whiteflies and not transmitted by seed or mechanical methods like sap transmission.

The reported minimum acquisition access period (AAP) and inoculation access period (IAP) of whiteflies in case of TYLCV ranged from 10 to 60 min (Cohen and Nitzamy, 1966; Mansour and Al-Musa, 1992; Brown and Bird, 1992; Mehta *et al.*, 1994; Brown, 1994). As per Zeidan and Czosnek (1991), different whiteflies given access to the same leaf for the same period of time acquired variable amounts of virus. *B. tabaci* could acquire an estimate of $0.5 \times 10^9$ virions (Polston *et al.*, 1990) or $6 \times 10^8$ virus genomes (Zeidan and Czosnek, 1991). Transmission of TYLCV and the bipartite begomovirus *Squash leaf curl virus* (SLCuV) by *B. tabaci* was found to be very efficient wherein a single whitefly was able to infect a tomato plant after 24 h AAP, and the efficiency of transmission reached cent per cent when 5–15 insects were used (Czosnek *et al.*, 2001). Brown and Czosnek and Ghanim (2002) reported that the frequency of virus transmission increased when AAP was increased. According to Czosnek and Ghanem (2012) the acquired TYLCV were retained in the whitefly for its entire life.

Ghanim *et al.* (2001) reported that the whiteflies required a latent period of one hour before the begomovirus ingested could be transmitted. They also proposed that during the latent period, the virus first got translocate through the midgut then to the haemolymph (insect blood) and finally to the salivary glands before it got secreted with saliva during feeding.

Rajasri *et al.* (2011) studied the whitefly transmission of ToLCV in tomato and reported that when 20 whiteflies provided with 24 h AAP and IAP were able to cause cent per cent transmission. Anandhan *et al.* (2011) studied the whitefly transmission of ToLCV and observed 84 per cent transmission using 20 whiteflies with 24 h AAP and IAP.

Tsering and Patel (1990) carried out a study in which virus free *B. tabaci* fed with infected bhendi yellow vein mosaic plants and then released to healthy okra in glasshouse trials. They found that 5 of 15 okra plants were infected with BYVMV. Sheikh *et al.* (2013) tested the efficiency of *B. tabaci* to transmit the BYVMD and found 50 per cent transmission of the virus to healthy okra plants. Singh *et al.* (2016) studied the transmission of BYVMV by various methods *viz.*, mechanical, grafting, aphid and whitefly transmission. The results indicated that *B. tabaci* could readily transmit BYVMV with cent per cent. However, the aphids (*Myzus persicae, Aphis gossypii* and *Aphis craccivora*) as well as mechanically could not transmit the virus.

According to Jose and Usha (2003), typical yellow vein symptoms developed in the okra plants 15 days after inoculation (DAI) with viruliferous whiteflies and they confirmed the presence of both DNA-A and DNA-β in the infected plants by southern blot hybridization technique. Venkataravanappa *et al.* (2015) also reported cent per cent disease incidence of BYVMD by the whitefly transmission and according to them, whiteflies released at the rate of 10 per seedlings was able to produce the disease within 8-12 days of incubation period.

### 2.3.2 Graft transmission

According to Reddy and Yaraguntaiah (1979), ToLCV could be transmitted through grafting and dodder. Singh *et al.* (2009) successfully did graft transmission of PYVMV and observed cent per cent transmission. Singh *et al.* (2016) and Naveen (2016) also confirmed cent per cent graft transmission of YVMD in healthy okra through wedge grafting. Ghevariya and Mahatma (2017) reported that the veneer and approach grafting were the best method of transmission of geminivirus like BYVMV.

### 2.3.3 Seed transmission

Weerasinghe (2010) reported that ToLCNDV ( in bittergourd) was transmitted only through whiteflies while seed and mechanical transmissions were not possible. Eui-Joon *et al.* (2016) and Manivannan *et al.* (2019) reported seed transmission of begomoviruses *viz., Tomato yellow leaf curl virus* and *Bittergourd mosaic virus* respectively.

## 2.4 CROSS-INFECTION STUDY

Muniyappa *et al.* (2003) and Singh *et al.* (2008) reported that PYVMV was not responsible for yellow vein disease in okra. Hamsa *et al.* (2016) proved the absence of cross-infection of PYVMV in tomato and okra seedlings through insect transmission of the virus as no symptoms were observed in any of the crops.

Prakasha (2010) reported whitefly transmission of BYVMV to pumpkin (cv. Big) seedling which produced vein clearing symptom after 15 to 20 days of inoculation. Other symptoms reported were vein netting at early stage and complete silvering at advanced stage.

## 2.5 IMMUNODETECTION

### 2.5.1 Enzyme linked immunosorbant assay (ELISA)

ELISA had been reported as easy and accurate immunological assay for the detection of geminiviruses especially begomoviruses (Roberts *et al.*, 1984; Thomas *et al.*, 1986; Aiton and Harrison, 1989; Muniyappa *et al.*, 1991; Harrison *et al.*, 1991; Swanson *et al.*, 1992). Muniyappa et al. (1991) used different monoclonal antibodies of *African cassava mosaic virus* (ACMV) and *Indan cassava mosaic virus* (ICMV) for the detection and characterization of ToLCV. Seepiban *et al.* (2017) studied the serological relation of *Tomato yellow leaf curl Thailand virus* with TAS-ELISA using monoclonal antibodies.

According to Harrison and Robinson (1999) BYVMV was morphologically and serologically related to ACMV, a geminivirus. Sheikh *et al.* (2013) detected the presence of Geminivirus by DAS-ELISA using *Okra yellow vein mosaic virus* (OYVMV) specific antiserum in which 86 of 100 samples gave a positive reaction.

### 2.5.2 Dot-immunobinding assay (DIBA)

Cancino *et al.* (1995) reported that DIBA was an efficient method for detection of geminiviruses like bean golden mosaic virus. Ghanem (2002) reported that serological detection of virus using DIBA was effective method. He could detect *Okra leaf curl virus* (OLCV), a begomovirus using OLCV-polyclonal antibody (PAb) in both naturally infected okra and the whiteflies inoculated plants. The results indicated the efficiency of DIBA for detection of geminiviruses, as well as other viruses (Makkouk *et al.*, 1993; James and Mukerji, 1996; Abdel-Salam, 1999 and Ghanim and Czosnek, 2000). Furthermore, OLCV-PAb differentiated non-viruliferous *B. tabaci* and viruliferous insects collected either from okra plants in field or in greenhouse trails.

Pun *et al.* (1999) successfully employed DAC-ELISA on nitrocellulose membrane to detect BYVMV in infected okra plants. Abou-Jawdah (2008) reported that detection of ToLCV could be done using DIBA on the inoculated susceptible cultivar using ACMV-PAB.

## 2.6 MOLECULAR DIAGNOSIS AND CHARACTERIZATION

### 2.6.1 Isolation of genomic DNA

Most common method of DNA isolation was cetyl trimethyl ammonium bromide (CTAB) method. DNA extraction of begomovirus causing BYVMD was performed by employing CTAB-Mucilage-free method (Ghosh *et al.*, 2009).

Ananthan *et al.* (2011) extracted total DNA of ToLCV using CTAB method (Dellaporta *et al.*, 1983) and dissolved the extracted DNA in distilled water instead of 1X TE buffer. Rajasri *et al.* (2011) extracted total nucleic acid of ToLCV by Gem-CTAB method (Rouhibaksh *et al.*, 2008).

Hamsa *et al.* (2016) extracted the total DNA from young pumpkin leaves infected with PYVMD using CTAB method. Total DNA isolated was quantified by biospectrophotometric method as given by Sambrook and Russel (2001).

### 2.6.2 Amplification of coat protein gene of BYVMV, ToLCV and PYVMV isolates

#### 2.6.2.1 Polymerase chain reaction (PCR)

PCR has been considered as the most rapid and sensitive method for the detection of begomoviruses (Varma and Malathi, 2003). The sequence analysis of CP gene region was recognised to be sufficient for the initial identification of Begomovirus (Fauquet *et al.*, 2003) and later suggested by the International Committee on Taxonomy of Viruses (ICTV, 8th Report, 2005). Use of CP gene

primers successfully detected Begomovirus by PCR in many cucurbits (Sohrab *et al.*, 2006; Singh *et al.*, 2007; Tiwari *et al.*, 2008). The CP gene primers were used for detection of Begomovirus isolates by PCR by Tiwari *et al.*, 2012.

PCR-amplification using Begomovirus-specific primers showed the presence of a Begomovirus component equivalent to DNA-A in diseased bhendi plants (Jose and Usha, 2000).

Deng *et al.* (1994) designed a set of primers specific to coat protein of Begomovirus to screen the presence of Begomovirus DNA in the diseased tomato leaf curl DNA samples and later these primers were widely used to detect the whitefly transmitted Geminivirus. The DengA/DengB primer pair targets a ~530 bp region that starts at the DNA-A origin of replication and continues to the 5'end of the AV1 (coat protein) gene. The sequences of the Deng universal primers were: DengA: 5' TAATATTACCKGWKGVCCSC 3' and DengB: 5' TGGACYTTRCAWGGB-CCTTCACA 3'. Reddy *et al.* (2005) did PCR amplification using begomovirus-specific primers DengA and DengB to detect Begomovirus.

Anandhan *et al.* (2011) amplified CP gene of ToLCV by PCR using the following primers, AV103F: 5'– ATGGTGAAGCGACCAGCAGAT-3' and AV104R: 5'–TTAATTTGTTACCGAATCTA-3' (Sivalingam and Varma, 2007). Naveen (2016) successfully employed AV/AC primer specific to coat protein of begomoviruses for the amplification of begomoviruses *viz.*, BYVMV and Bittergourd mosaic virus (BGMV).

**2.6.2.2 Gel electrophoresis**

Tiwari *et al.* (2012) did analysis of PCR products by gel electrophoresis in 1.2 per cent agarose gel with DNA marker.

### 2.6.3 Characterization of coat protein gene of BYVMV, ToLCV and PYVMV

The CP gene of pumpkin yellow vein mosaic infected plant samples had 98% nucleotide sequence similarity with *Squash leaf curl China virus*- [Pumpkin Coimbatore] segment A (AY184487.3) and 97% similarity with *Squash leaf curl China virus*- [Pumpkin: Lucknow] segment A (DQ026296.2) in BLAST. The sequence also showed more than 90% of similarity to *Tomato leaf curl New Delhi virus* (Hamsa *et al.*, 2016). Vanthana *et al.* (2017) reported that the CP gene amplification of ToLCV isolate from Nepal shared highest sequence similarity of 94 per cent to *Tomato leaf curl Gujarat virus* isolate.

Prakasha (201-) reported that the full CP gene of BYVMV isolate from Karnataka comprising of 771 bp shared highest nucleotide (99.35 %) and amino acid (99.65 %) sequence identity with BYVMV Madhurai Variant 3 (AJ278861) and Barrackpur isolate (EF417918). Naveen (2016) reported that the sequenced CP gene of BYVMV isolate expressed maximum similarity to *Okra enation leaf curl virus* (OELCV).

### 2.6.4 Full genome amplification of DNA-A of BYVMV, ToLCV and PYVMV

#### 2.6.4.1 Rolling circle amplification (RCA)

In 2004, RCA using phi29 DNA polymerase was first applied to viral genomes. Rector *et al.* (2004) showed that the papillomavirus genome, which is composed of circular double-stranded DNA, could be efficiently amplified from tissue samples using RCA. At the same time, Inoue-Nagata *et al.* (2004) used this method for cloning of a single-stranded circular DNA genome segment of a geminivirus.

Detection and characterization of geminiviruses have become easier with the application of φ-29 DNA polymerase, which selectively amplifies the circular DNA through RCA (Inoue-Nagata *et al.*, 2004; Haible *et al.*, 2006; Knierim and Maiss,

2007). Small circular DNA are preferentially amplified by RCA, what makes geminiviruses, which have circular single stranded (ss) DNA genome, ideal substrates for RCA (Jeske *et al.*, 2001).

RCA is an isothermal method which uses the DNA polymerase of the *Bacillus subtilis* bacteriophage phi29 (Φ29), a monomeric enzyme with a molecular mass of about 66 kDa, that possesses a polymerase activity located on the C-terminal domain and two degradative activities: pyrophosphorolysis (depolymerization), a process for which its physiological significance is still unclear, and a 3'–5'-exonuclease activity within its N-terminal domain, involved in proofreading function, resulting in an error rate of only 1 in $10^6$ –$10^7$, approximately 100 times more accurate than Taq DNA polymerase, which gives an error rate of 1 error in $10^4$ – $10^5$ amplified base pairs (Eckert and Kunkel, 1990). The enzyme has two other intrinsic properties: high efficiency (70 kb) and strand displacement ability making unnecessary the participation of accessory proteins and DNA helicases, thus allowing templates as small circular ssDNA to be replicated to nearly unlimited extent using the rolling circle replication mechanism (Blanco *et al.*, 1989; Garmendia *et al.*, 1992; Blanco and Salas, 1996; Dean *et al.*, 2001).

Wyant *et al.* (2011) and Rishishwar *et al.* (2015) subjected the samples to RCA (Haible *et al.*, 2006) using TempliPhi Kit (GE Healthcare, formerly Amersham) followed by restriction digestion of RCA product with restriction enzymes *viz.*, *Hpa*II or *Bfu*CI.

**2.6.4.2 Restriction with different endonucleases**

The RCA methodology in combination with RFLP recently had been suggested by Haible *et al.* (2006) to be a highly reproducible tool for geminivirus diagnosis. According to him, the diagnosis of geminiviral (Begomovirus) infection by using RCA/RFLP is largely independent of source plant type and origin, viral

genome organization and sample preparation method, since neither specific primers nor expensive equipment, like thermocycler were needed for the reaction.

The ssDNA genome of the Begomovirus isolates when amplified by RCA followed by digestion with *Xba*I enzyme yielded ~2.7 kb full genome (Briddon *et al.*, 2002). Kirthi *et al.* (2002) purified Repricative Form (RF) of DNA of *Tomato leaf curl Bangalore virus* (ToLCBV [Ban 5]) and it was subjected to restriction digestion with hexacutters like *EcoRI, HindIII, Pst I or BamHI.* The RCA product of ToLCV was restricted with *BamHI, HindIII, XbaI and KpnI* restriction endonucleases which were commonly seen as unique restriction sites in Begomovirus genome that gives 2.7 kb (either DNA-A or DNA-B) (Rajasri *et al.*, 2011). Jyothsna *et al.*, (2013) digested the RCA products of RF DNA of Begomovirus infecting potato with different endonucleases (*BamHI, EcoRI, HindIII, KpnI, and XbaI*) and the ~2.7 kb fragments representing full-length DNA-A and DNA-B components and ~1.3 kb fragments representing alpha-satellites or beta-satellites generated were purified and cloned into pUC18.

**2.6.4.3 Development of primers for full genome amplification of DNA-A**

Two methods were used commonly to clone the genome of viruses. The first method was based on PCR using universal or specific primers, amplifying either partially or completely the genome (Patel *et al.*, 1993). The second method was based on the extraction of total DNA or semi-purified DNA enriched with the replicative form of the viral genome, followed by digestion with restriction enzymes, southern blot hybridization and cloning after digestion with a single cutter enzyme (Gilbertson *et al.*, 1991; Srivastava *et al.*, 1995). This procedure usually has low efficiency due to the low concentration of viral genome in the preparation.

Patel *et al.* (1993), Singh *et al.* (2008), Venkataravanappa *et al.* (2012, 2013 a, 2013 b) and Rishishwar *et al.* (2015) developed three sets of overlapping primers

specific to each begomovirus to amplify the whole genome under the study using sequences of the begomoviruses obtained from NCBI database.

Jose and Usha (2003) isolated BYVMV DNA-A and initially amplified it using begomovirus DNA A-specific primers, and then the 2.7 kb amplified product was cloned (pBY1) and sequenced.

**2.6.4.4 Extraction of DNA from gel**

Briddon *et al.* (2002) used MinElute® gel extraction kit for eluting RCA products (Qiagen, Hilden, Germany). Tiwari *et al.* (2012) did molecular characterization of begomoviruses in cucurbits (pumpkin) and the PCR products were eluted using elution kit (Wizard SV gel and PCR clean up system, Promega, USA). Venkettaravanappa *et al.* (2015) electrophoresed the digested DNA fragments of BYVMV and DNA-B-like sequence of ToLCNDV in 1 per cent low melting agarose gel and then the fragments were purified using QIAquick gel extraction kit (QIAGEN Inc., USA). Amplification products were purified from gel using DNA gel extraction kit (Axygen Biosciences, USA) by Hamsa *et al.* (2016). The amplified products were analyzed by electrophoresis in an agarose gel (1%) and further purified (Jeevalatha *et al.*, 2017).

**2.6.5 Characterization of full genome of DNA-A**

Namratha *et al.* (2010) used Basic Local Alignment Search Tool (BLAST) program at the NCBI website (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.*, 1990) to find the related begomovirus sequences for the characterization of the virus. Tiwari *et al.* (2012) deposited the obtained begomovirus sequence in GenBank database. They did BLAST search analysis of nucleotide sequence of the virus isolates using BLASTn (optimised for somewhat similar sequences) with sequences available in the GenBank database (http://BLAST.ncbi.nlm. nih.gov/BLAST.cgi?PAGE).

In India, BYVMD is caused by *Bhendi yellow vein mosaic virus* (BYVMV) and a beta-satellite (Jose and Usha, 2003). Venkataravanappa *et al.* (2013a) characterized the okra samples showing yellow vein mosaic disease symptoms from Bangalore, Karnataka and reported 96.1 per cent nucleotide similarity with *Bhendi yellow vein mosaic virus*, 92.8 per cent with *Cotton leaf curl Bangalore virus* and 81.1 to 86.2 per cent to *Okra enation leaf curl virus*. *Okra yellow vein mosaic virus* (OYVMV) has also been reported from BYVMD affected okra (Taware *et al.*, 2010). In addition, *Mesta yellow vein mosaic virus* (MeYVMV) infecting fibre crop Mesta was also found to be associated with BYVMD in okra from northern India (Zaffalon *et al.,* 2012). Recently, two other reports; the bipartite begomovirus, *Bhendi yellow vein Delhi virus* (BYVDV, Venkataravanappa *et al.*, 2012) and *Bhendi yellow vein Bhubaneswar virus* (BYVBV, Venkataravanappa *et al.,* 2013b), both of which are associated with symptoms resembling BYVMD in okra were reported.

In India, tomato leaf curl disease is caused by four recognised begomovirus species, as demonstrated by Koch's postulates. They are two bipartite, *Tomato leaf curl New Delhi virus* (ToLCNDV) (Padidam *et al.,* 1995; Srivastava *et al.*, 1995; Tripathi and Varma, 2003; Sivalingam *et al.,* 2004) and *Tomato leaf curl Gujarat virus* (ToLCGV) (Chakraborty *et al.*, 2003) and two monopartite, *Tomato leaf curl Bangalore virus* (ToLCBV) (Sivalingam *et al.,* 2004) and *Tomato leaf curl Karnataka virus* (ToLCKV) (Chatchawankanphanich and Maxwell, 2002)

Two species of geminiviruses with bipartite genome, causing PYVM had been reported from India. *Tomato leaf curl New Delhi virus-India* (Maruthi *et al.*, 2007) had been reported from North India and *Squash leaf curl China virus-India* from South India. Later, bipartite *Squash leaf curl Palampur virus* was also reported to be associated with PYVM (Jaiswal *et al.*, 2011). Namratha *et al.* (2010) did the complete sequencing of DNA-A of begomovirus isolated from pumpkin with 2756 nt. In BLAST search, the complete sequence of DNA-A had 99% nucleotide sequence

identity with *Tomato leaf curl Palampur virus*. Tiwari *et al.* (2012) reported that two different begomoviruses: ToLCNDV on pumpkin (AM286434: unpublished), and SLCCNV in pumpkin (AY184487: Muniyappa *et al.* (2003); DQ026296: Singh *et al.* (2007)) had been identified in India by sequence analysis of complete DNA-A genome causing PYVMD.

## 2.7. GENETIC DIVERSITY AND PHYLOGENY OF VIRUS

According to Archer (2009) the use of DNA and amino acid sequences to estimate evolutionary history has denominated molecular phylogenetics. Thus, molecular characteristics were used to classify organisms and placing them on a map of evolutionary relationships known as the phylogenetic tree that shows the probable evolution of various organisms. The Neighbor joining (NJ) is one of various methods to reconstruct phylogenetic trees (Saitou and Nei, 1987; Tamura *et al.*, 2004).

### 2.7.1 Genetic diversity and phylogeny of coat protein gene

Hamsa *et al.* (2016) reported that the phylogenetic analysis of coat protein of PYVMD begomovirus revealed that the virus isolates was found to be more related to *Squash leaf curl China virus*- [Pumpkin: Lucknow] compared to *Tomato leaf curl New Delhi virus* though they clustered in a single clade. Naveen (2016) reported that the phylogenetic analysis of CP of BYVMV and *Bittergourd mosaic virus* were clustered separately and hence distantly related to each other.

### 2.7.2 Genetic diversity and phylogeny of DNA-A of BYVMV, ToLCV and SLCCNV

Kirthi *et al.* (2002) and Reddy *et al.* (2005) did multiple alignment of DNA A sequences of ToLCBV strains (ToLCBV [Ban 1], ToLCBV [Ban 4], ToLCBV [Ban 5] and ToLCBV [Kolar]) and ToLCKV used the Clustal W (1.7) programme (Wisconsin Package). Phylogenetic analyses were performed by parsimony and distance methods

by using the software PAUP version 4.0 b10 (Altivec) (Swofford *et al.,* 1998). The heuristic and neighbour-joining method of search was employed for generating phylogenetic trees. Bootstrapping for 1000 replications was performed by stepwise sequence addition and tree-bisection-reconnection. Anandhan *et al.* (2011) constructed phylogenetic tree using Clustal X and Tree View 1.6.5 software.

Tiwari *et al.* (2012) in his study did sequences alignment using Clustal W algorithm (Thompson *et al.,* 1994) and MEGA-4 version (Tamura *et al.,* 2007); and bootstrapping for 1000 replicates was used for generating phylogenetic trees. Venkataravanappa *et al.* (2012) analysed nucleotide and amino acid sequence homologies using BioEdit version 7.0 (Hall, 1999). Multiple alignments of sequences were done using Clustal W program (Thompson *et al.,* 1994], and phylogenetic trees were constructed with MEGA 4.0 software (Tamura *et al.,* 2007) using neighbor joining methods. Statistical support for clades was estimated using 1,000 bootstrap interactions. They found that the characterized geminivirus isolated from okra showing vein clearing symptoms clusters with ToLCNDV and the phylogenetic analysis revealed that the isolate was distinct from all other geminiviruses group that reported from other vegetable crops.

Jose and Usha (2003) reported that the phylogenetic relationship of BYVMV isolate of India determined using Clustal W revealed to be most closely related to the Pakistan isolate of BYVMV (Acc. No. AJ002453; 92.5% identity) and less so to OYVMV (Acc. No. AJ002451; 89 % identity) and a Pakistan isolate of CLCuV (Acc. No. AJ002455; 82 % identity).

PYVMD was associated with two begomoviruses, *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Squash leaf curl China virus* (SLCCNV) (Maruthi *et al.,* 2003; Singh *et al.,* 2008). Venkataravanappa *et al.* (2012) reported that the characterized geminivirus isolated from okra showing vein clearing symptoms clusters with ToLCNDV. The phylogenetic analysis revealed that the isolate was

distinct from all other geminiviruses group that was reported from other vegetable crops.

According to Namratha *et al.* (2010), infection of a new Begomovirus namely, *Tomato leaf curl Palampur virus* (ToLCPMV), and a beta-satellite identified as *Pepper leaf curl beta-satellite (*PepLCB) in pumpkin led to development of typical symptoms of the yellow vein mosaic disease and phylogenetic analysis revealed that the virus was distantly related to other PYVMD causing begomoviruses.

## 2.8 DEVELOPMENT OF SPECIFIC PRIMERS

Sivalingam and Varma (2007), developed specific primers for ToLCD using the full-length DNA-A sequences of begomoviruses associated with ToLCD in India, retrieved from GenBank, NCBI database and then analysed by Clustal W software. Based on nucleotide sequence alignment, the conserved and common regions of different begomoviruses associated with ToLCD in India, were identified. The primers were designed accordingly, and synthesized at Genetix Biotech, Germany. The virus specific primers were validated by specific amplification from the DNA-A clones of ToLCNDV (pUTDV-A), ToLCBV (pUTBV-A) and intergenic region (IR) of ToLCGV (pGTUP-IR). Zhou *et al.* (2008) developed specific primers for detection of the virus using the already existing sequences of the virus in NCBI database. They analysed the different ORF's of the viruses to identify region of maximum variability and then designed specific primers from that particular region.

# *Materials and Methods*

# 3. MATERIALS AND METHODS

The present research work entitled 'Diversity of begomoviruses infecting major vegetable crops" was carried out in the Department of Plant Pathology ,College of Agriculture, Vellayani during the period 2017-2019, with the objectives to identify the genetic variability in begomoviruses isolates infecting okra, tomato and pumpkin; and the mode of transmission of the virus in these crops. The materials and methods adopted for conducting the study were presented in this chapter.

## 3.1 COLLECTION OF BEGOMOVIRUS INFECTED OKRA, TOMATO AND PUMPKIN

The study was conducted in three major vegetable growing taluks of Thiruvananthapuram district *viz.,* Neyyattinkara, Nedumangad and Chirayinkeezhu; and Kollam, Kottarakara and Pathanapuram (Punalur) taluks of Kollam district (Plate 1). Leaves and plant samples exhibiting leaf curling, purpling, and/or stunting were collected from the major tomato production areas; and leaves and plant samples showing vein clearing, vein thickening, yellowing and stunting were collected from okra and pumpkin growing fields in the above-mentioned areas of southern Kerala. Samples were collected from feild by stratified random sampling method. Infected plants were randomly selected from the fields and disease scoring was done after modification on the 0-5 scale developed by Bos (1982). The vulnerability index (V.I.) and disease incidence (D.I.) in each crop was calculated from all the locations. Bhendi yellow vein mosaic disease was scored as per the following score chart (Naveeen, 2016) (Plate 2):

0 – No symptom

1 – Very mild mottling, initial vein clearing

2 – Mottling and complete yellowing of veins with green interveinal region

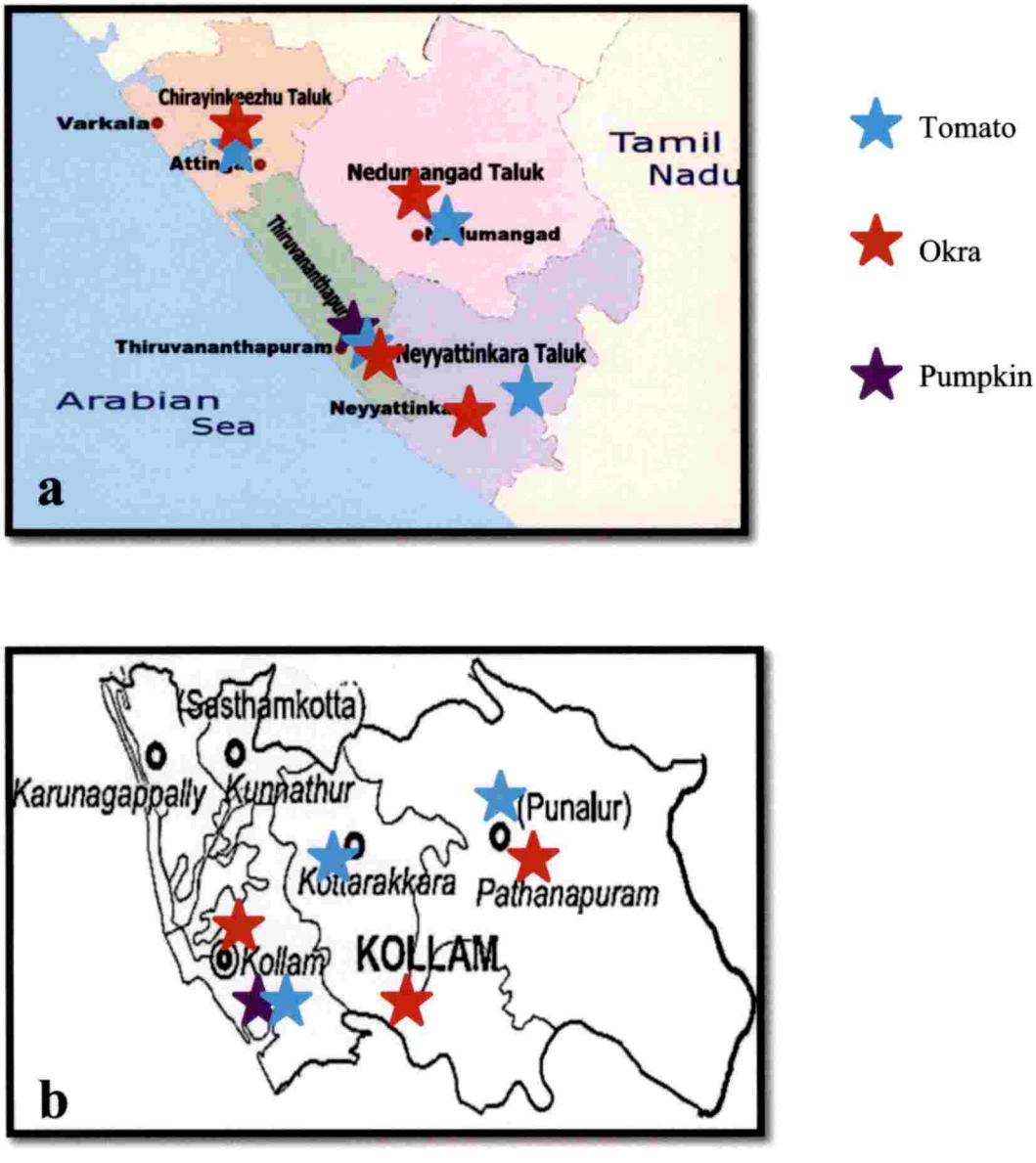



Plate 1. Location maps of sample collection of the Begomovirus infected okra, tomato and pumpkin plants. (a) Thiruvananthapuram district with locations *viz.* College of Agriculture (Vellayani), Neyyattinkara, Nedumangad and Chirayinkeezhu and (b) Kollam district with locations *viz.* Kollam, Kottarakkara and Punalur.

0- No symptom

1 -Very mild mottling, initial vein clearing

2 - Mottling and complete yellowing of veins, interveinal regions remain green

3 – Blisters and raised surfaces on leaf

4 - Distortion of leaves, whole leaf become yellow and reduction in leaf size

5 -Stunting with negligible or no flowers, yellowish and deformed fruits

Plate 2. Vulnerability index of okra yellow vein mosaic disease as described by Bos (1982) with slight modification (score 0-5) (Naveen, 2016)

3 – Blisters and raised surfaces on leaves

4 – Distortion and curling of leaves with reduction in leaf size

5 – Stunting with negligible or no flowering and deformed fruits

Score chart for tomato leaf curl disease was as follows (Plate 3):

0 – No symptom

1 – Yellow specks on leaves

2 – Initial leaf curling and chlorosis

3 – Severe leaf curling and blistering of leaf lamina

4 – Purplish discoloration and reduced leaf size

5 – Stunted growth with negligible or no flowering, distorted small sized leaves and fruits

The score chart followed for pumpkin yellow vein disease was as follows (Plate 4):

0 – No symptom

1 – Initial vein clearing

2 – Slight mottling and complete yellowing of veins, interveinal regions remain green

3 – Complete chlorosis and mild blistering

4 – Distortion of leaves, blisters and raised surfaces on leaves and reduction in leaf size

5 – Stunting with negligible or no flowering

Based on the scoring for each infected plants the V. I. was calculated for the samples using the formula,

$$V.I. = \frac{(0n_0+1n_1+2n_2+3n_3+4n_4+5n_5) \times 100}{n_t(n_c-1)}$$ (Bos, 1982)

0 –No symptom

1 –Yellow specks on leaves

2 – Initial leaf curling and chlorosis

3-Severe leaf curling and blister development of leaves

4-Purplish discolouration

5-Stunted growth with negligible or no flowering, distorted small sized leaves and fruits

Plate 3. Vulnerability index of tomato leaf curl disease as described by Bos (1982) with slight modification ( score 0-5)

0-No symptom

1-Initial vein clearing

2-Slight mottling and complete yellowing of veins, interveinal regions remain green

3-Complete chlorosis and mild blistering

4-Distortion of leaves, blisters and raised surfaces on leaves and reduction in leaf size

5-Stunting with negligible or no flowering

Plate 4. Vulnerability index of pumpkin yellow vein mosaic disease as described by Bos (1982) with slight modification ( score 0-5)

V.I. – Vulnerability index

$n_0, n_1 \ldots n_5$ – Number of plants in disease category of 0, 1,2, 3, 4 and 5

$n_t$ – Total number of plants

$n_c$ – Number of categories

The percentage disease incidence was assessed by the number of plants expressing disease symptoms and the total number of plants examined by using the formula.

$$\text{Disease incidence (D.I.)} = \frac{\text{Number of plants infected} \times 100}{\text{Total number of plants examined}}$$

During the survey, data on the crop variety and season of maximum disease incidence were recorded.

## 3.2 SYMPTOMATOLOGY

Infected okra, tomato and pumpkin plants showing begomovirus infection were collected from field and the various symptoms expressed by them were studied. Days taken for symptom development and the nature of the symptom developed were noted in case of the three diseases *viz.*, Bhendi yellow vein mosaic disease (BYVMD), Tomato leaf curl disease (ToLCD) and Pumpkin yellow vein mosaic disease (PYVMD).

## 3.3 TRANSMISSION STUDIES

Generally, the begomoviruses were transmitted by whiteflies and through graft transmission. Hence, studies were conducted to transmit the begomoviruses infecting okra, tomato and pumpkin through both the methods. Since seeds were the sole source of propagation in these three crops, seed transmission was also tested on seeds collected from the virus infected plants.

3.3.1 Insect transmission

The healthy culture of the whiteflies, *Bemisia tabaci* Gen. (F: Aleyrodidae, O: Homoptera) reared on disease free brinjal plants kept inside insect proof cages were used for the transmission of the begomoviruses (Plate 5). Adult flies were collected from the brinjal plants using an aspirator and released on the infected plants for acquiring the virus for about 24 h (acquisition access period). Prior to inoculation of the viruses, a latent period of 1 h was provided since all the three viruses under study follows a circulative-persistent mode of transmission in the whitefly vector. The viruliferous whiteflies were then collected using aspirator and released onto healthy okra, tomato and pumpkin seedlings of about 2-3 leaf stage for inoculation of the viruses.

Whiteflies were released on healthy seedling at the rate of 20 viruliferous whiteflies per seedling for 24 h for the inoculation / transmission of the viruses (inoculation access period) (Table 1).

The whiteflies were killed after 48 h by spraying with an insecticide (Imidacloprid 0.05 %). The inoculated seedlings were kept inside the insect proof cages and were observed for the development of symptoms. The days taken for symptom development, disease incidence and vulnerability index were recorded for each of the virus transmission.

3.3.2 Graft transmission

Graft transmission study of viruses was done by wedge grafting wherein infected twigs were taken as scion and 5-6 leaved healthy seedlings were used as rootstock for the grafting. Apical portion of the rootstocks were removed in such a way that a minimum of two nodes still remains intact to the rootstock. A longitudinal slit was made on the rootstock so that the "V" shaped end of the scion could be inserted in them. The graft union was then made tight using either a grafting clip or

Table 1. Insect transmission of BYVMV, ToLCV and PYVMV using whiteflies (*B. tabaci*)

| Sl. No. | Virus | No. of whiteflies released | Acquisition access period (AAP) (h) | Latent period (h) | Inoculation access period (IAP) (h) |
|---|---|---|---|---|---|
| 1. | BYVMV | 20 | 24 | 1 | 24 |
| 2. | PYVMV | 20 | 24 | 1 | 24 |
| 3. | ToLCV | 20 | 24 | 1 | 24 |

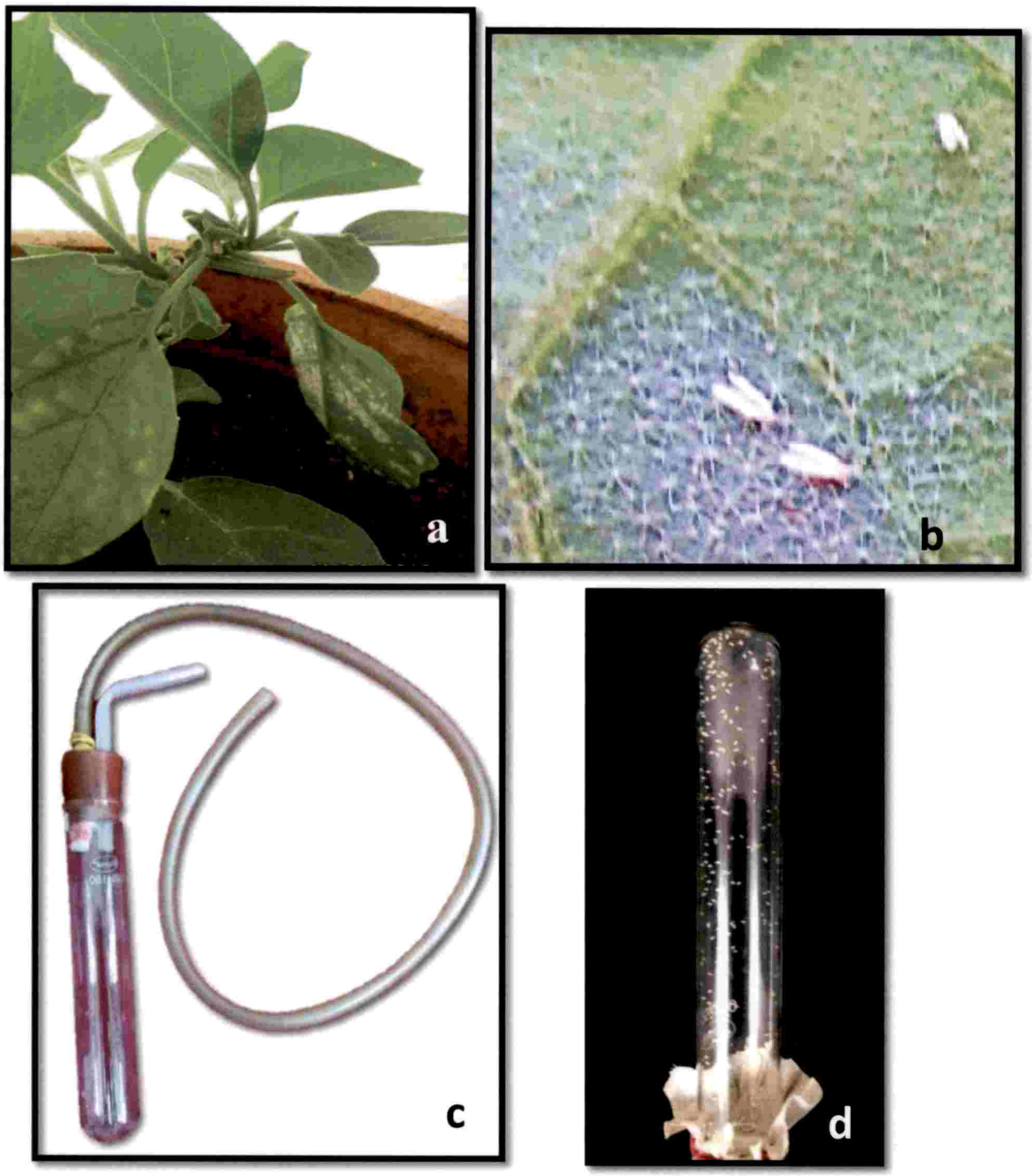



Plate 5. Whitefly transmission of Begomoviruses. (a) Whiteflies being reared on brinjal plant, (b) Vector – Whiteflies (*Bemisia tabaci* Genn.), (c) Aspirator and (d) Whiteflies collected in collection tube of aspirator for insect transmission of Begomoviruses.

grafting tape. These grafted seedlings were then kept inside insect proof cages. Observation on days taken for symptom development, percentage disease incidence and vulnerability index were recorded.

3.3.3 Seed transmission

Seeds collected from the begomoviruses infected okra, tomato and pumpkin plants were sown in pots kept inside the insect proof chambers for further growth. Ten seedlings of each crops were then closely observed for more than two months for the development of symptom. The observation on the days taken for development of symptom (if any), percentage disease incidence and vulnerability index were noted for each of the diseases.

3.4 CROSS-INFECTION STUDY

Since the begomoviruses could neither be transmitted mechanically nor grafting was possible among plants belonging to different family, the only method applicable for cross-infection study was through insect transmission using whiteflies. Hence, viruliferous whiteflies taken from BYVMV infected okra plants were allowed to feed on 2-3 leaved tomato and pumpkin seedlings under insect proof condition. Similarly, ToLCV and PYVMV acquired whiteflies were fed on healthy okra and pumpkin seedlings and okra and tomato seedlings respectively under insect proof condition. The days taken for symptom development (if any), percentage disease incidence and vulnerability index were noted.

3.5 IMMUNODETECTION

3.5.1 Immunodetection by Enzyme linked immunosorbent assay (ELISA) method

Immunological characterization of the infected okra, tomato and pumpkin plants were done by using Double Antibody Sandwiched Enzyme Linked

Immunosorbent Assay (DAS-ELISA) using polyclonal antiserum specific to *Tomato leaf curl New Delhi virus* (ToLCNDV) and Triple Antibody Sandwiched Enzyme Linked Immunosorbent Assay (TAS-ELISA) using monoclonal antiserum specific to *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV). These antisera were used to detect the presence of virus and study the serological relationship as they were the only antisera available to test the presence of the respective begomoviruses in the samples. DAS-ELISA and TAS-ELISA were performed using the protocol prescribed by the antisera provider (DSMZ, Germany) with three replications of each healthy, infected sample and blank.

The protocol followed for DAS-ELISA was as follows: The specific antibody was first diluted in coating buffer, i.e. 20 µl in 20 ml buffer at a recommended dilution of 1:1000, then about 200 µl of the antibody diluted in coating buffer was added to each well of the microtitre plate (TARSONS, 96 Well micro test plate) with three replications for each treatment. The plate was then covered using aluminium foil and was kept for incubation at 37 °C for 2-4 h. Then the plate washed with PBS-Tween thrice with 2 min incubation using ELISA plate washer (ImmunoWash™ 1575 Microplate Washer, BIO-RAD). Two hundred microlitre of the test samples extracted in begomovirus extraction buffer in the ratio 1:20 (w/v) were added to duplicate wells. The plate was covered and kept for incubation overnight at 4 °C. The following day, after the plate was washed using PBST as mentioned earlier, enzyme conjugate (recommended dilution of 1:500) in the conjugate buffer was added to the wells at the rate of 200 µl per well. Again, the plate was covered and kept for incubation at 37 °C for 2-4 h. The plate was then washed with PBST as was done earlier. To the plate 200 µl aliquots of freshly prepared substrate (1 mg / ml p-nitrophenyl phosphate in substrate buffer) was added to each well. Further, the plates were covered and incubated at 37°C for 30-60 min (or as long as necessary to obtain clear reactions) in incubator. The result was then assessed using ELISA reader (iMark™ Microplate Reader, BIO-RAD) (Appendix I).

Immunodetection was also done using *African cassava mosaic* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) antisera by TAS-ELISA method. The protocol followed was as follows (Clark and Adam, 1977):

The specific antibody was diluted in coating buffer (i.e. 20µl in 20 ml buffer) at a recommended dilution of 1:1000. About 200 µl of this diluted antibody was then added to each well of the microtitre plate with three replications of each treatment. The plate was then covered and kept at 37°C for 2- 4 h in incubator. The plate was then washed with PBS-Tween using ELISA plate washer thrice with 2 min incubation in between each wash. After washing 200 µl of 2 per cent skimmed milk (spray dried milk) in PBS-Tween was added to each well (blocking). The plate was then covered and kept for incubation for 30 min at 37°C. The blocking solution was removed and plate was then washed as mentioned earlier. Two hundred microlitre of the test samples extracted in begomovirus extraction buffer in the ratio 1:20 (w/v) were added to duplicate wells. The plate was then covered and kept for incubation overnight at 4 °C. The plate was washed as mentioned earlier. Further, 200 µl of the antibody was added in appropriate (1:1000) conjugate buffer to each well. The plate was covered and incubated at 37 °C for 2- 4 h. The plate was then washed as before. Diluted RAM-AP (1:1000) in conjugate buffer, i.e., 20 µl in 20 ml buffer was added at the rate of 200 µl per each well. The plate was then covered and incubated at 37 °C for 1 h. The plate was washed as mentioned earlier. Finally, 200 µl aliquots of freshly prepared substrate (1 mg/ ml *p*-nitrophenyl phosphate in substrate buffer) was added to each well and the plate was covered and incubated at 37°C for 30-60 min. The absorbance was read at 405 nm in an ELISA reader. In case, there was two or more than two fold increase in the absorbance value of the disease sample compared to healthy sample, then begomovirus infection was confirmed. Otherwise sample was considered healthy.

Seeds of infected plants were also subjected for immunodetection in order to find seed transmission of the virus. The procedure for detection was the same as that mentioned earlier, except instead of leaf samples, seeds were taken from infected plants. Further, the optical densities of the diseased and healthy seed samples were analyzed for the detection of the virus. Those samples which gave an absorbance value twice or more than twice that of healthy samples were considered infected while those with absorbance value less than two fold of the healthy samples were considered healthy (uninfected).

3.5.2 Immunodetection by Dot immunobinding assay (DIBA)

Immunodetection was also done using Dot immunobinding assay (DIBA) using antisera specific to *Tomato leaf curl New Delhi virus* (ToLCNDV). The protocol followed was as follows: One gram of leaf sample was homogenized in 5 ml of antigen extraction buffer and filtered through muslin cheese cloth. 800 µl of the filtrate was transferred into 1.5 ml eppendorf tube and 400 µl of chloroform was added to it. The mixture was vortexed and centrifuged at 12000 rpm for 2 min. From this eppendorf tube 200 µl of the supernatant was pipetted out and 800 µl of begomovirus antigen extraction buffer was added to it and vortexed. Nitrocellulose membrane (NCM) of 3 cm long was cut and marked into squares of 1x1 $cm^2$ size and floated in Tris Buffer Saline (TBS) and then air dried it. The centre of squares was marked with a pencil and 10 µl of sample was spotted on the mark. The treated NCM was incubated for 30 min in room temperature and allowed to dry. It was soaked in TBS buffer and it was air dried. The NCM was then soaked in blocking solution with gentle oscillation for 1 h at room temperature using an oscillator (ROCKYMAX, TARSONS). Then the NCM was again rinsed in TBS buffer thrice with 10 min interval each and air dried. The NCM was floated in primary antibody of ToLCNDV, which was diluted at 1:200 dilution in TBS-Spray dried milk (TBS-SDM) and incubated overnight at $4^0$C. NCM was again rinsed with TBS buffer thrice as before

and air dried. This was followed by the addition of secondary antibody (Alkaline phosphatase conjugate) diluted at 1:200 dilution in TBS-SDM and incubated for 1 h. The NCM was then rinsed with TBS buffer thrice followed by air drying. Then the NCM was floated in freshly prepared solution of nitro blue tetrazolium (NBT) and bromo chloro indolyl phosphate (BCIP) in substrate buffer and kept in dark for 30 min incubation for colour development. After colour development, NCM was rinsed in fixing solution for 10 min, air dried between Whatman filter paper sheets and the intensity of colour developed was observed in Gel Doc system (Molecular Imager Gel DOC ™ XR+ with ImageLab™ Software, BIO-RAD) (Appendix II).

## 3.6 MOLECULAR DIAGNOSIS AND CHARACTERIZATION

Molecular diagnosis was done using PCR for the detection of the three begomoviruses *viz. Bhendi yellow vein mosaic virus* (BYVMV), *Tomato leaf curl virus* (ToLCV) and *Pumpkin yellow vein mosaic virus* (PYVMV) infecting okra, tomato and pumpkin plants respectively.

### 3.6.1 Isolation of genomic DNA

The genomic DNA of BYVMV, ToLCV and PYVMV were isolated using modified cetyl trimethyl ammonium bromide (CTAB) method (Lodhi *et al.*, 1994) as well as by using DNeasy plant mini kit (QIAGEN: Cat. No. 69104).

Protocol for genomic DNA isolation using modified CTAB method was as given below: 100 mg of fresh leaf tissue with characteristic symptoms was taken in a sterile mortar, liquid nitrogen was added to it to freeze the sample and powdered using a sterile pestle. The powdered samples were then mixed well with 1 ml of preheated 60°C extraction buffer (CTAB). 750 µl of these samples were transferred to a micro centrifuge tube and heated at 60°C for 30-45 min in a heating block. The samples were then centrifuged at 10000 rpm for 10 min at 4°C in centrifuge (Centrifuge 5804 R, eppendorf). The supernatant was taken and 10 µl of RNAase was

added to the supernatant and incubated for 37°C for 1 h. Equal volume (500 µl) of chloroform: isoamyl alcohol (24:1 mixture) was added to it and centrifuged at 15000 rpm for 10 min. The supernatant was then pipetted out and transferred to a micro centrifuge tube and 1 ml of ice cold 100 per cent ethanol was added (in equal volume basis), and incubated for 1h at -20°C. It was then centrifuged at 15000 rpm at 4°C for 10 min. Supernatant was then discarded and the pellet was washed with 1 ml of 70 per cent ethanol twice. It was then centrifuged at 12000 rpm for 5 min. The pellet was air dried to remove the ethanol. The dried pellet was suspended in 50 µl of 1X TE buffer and stored at - 20°C (Appendix III).

The genomic DNA was also isolated using DNA isolation using DNeasy plant mini kit (QIAGEN: Cat. No. 69104) as per the manufacturer's protocol.

The quality and quantity of DNA was measured using spectrophotometer (BioSpectrometer, eppendorf) at 260 and 280 nm. The protocol used for analyzing the quality of DNA was as follows: The 10 µl extracted DNA sample was diluted in 1000 µl of distilled water (so dilution factor was 100). The quality of DNA obtained was calculated as ratio of O.D. 260 to O.D. 280 value, for high quality DNA sample the ratio of O.D. 260 to O.D. 280 value was found to be 1.8. The reading at 280 nm gave the amount of protein in the sample while reading at 260 nm gave the quantity of DNA.

For single-stranded DNA (ssDNA), 1 O.D. at 260 nm = 20-33 ng $\mu l^{-1}$ of ssDNA. Hence, quantity of ssDNA was calculated as, O.D. 260 nm value x 33 ng $\mu l^{-1}$.

### 3.6.2 Amplification of coat protein gene

#### 3.6.2.1 Polymerase chain reaction analysis

For initial detection of the begomoviruses, PCR was performed from the total DNA using two sets of primers specific to coat protein (CP) region of the

begomoviruses *viz.*, AV/AC and DENG Forward and Reverse (Table 2). The synthesized primers being in powdered form were diluted to a final concentration of 10 pM with diethyl polycarbonate (DEPC) water prior to its use.

The PCR amplification reactions were carried out in a 25 µl reaction mixture containing 10X PCR Taq buffer - 5.0 µl, 1.5 mM $MgCl_2$ - 1.5 ml, 10 mM dNTPs (dATP, dGTP, dCTP and dTTP) - 0.5 µl, 3.0 µl DNA, 0.3 µl (2.5 Units) Taq polymerase enzyme, forward and reverse primers (5 pM) 1 µl each (SIGMA-ALDRICH) and 12.7 µl sterile distilled water. The reaction mixture was run in a thermocycler (Veriti 96 well Thermal Cycler, Applied Biosystems) under the specified conditions (Table 3).

### 3.6.2.2 Agarose gel electrophoresis of PCR products

Agarose gel of 1.2 per cent was prepared in 0.5X TAE buffer containing 0.5 µg $ml^{-1}$ ethidium bromide (EtBr). 2 µl of 6X loading dye was mixed with 10 µl of PCR products, loaded and electrophoresis was performed at 75V $cm^{-1}$ power supply with 0.5X TAE as electrophoresis buffer for about 1-2 h. The molecular standard used was 100 bp DNA ladder (GeNei). The gels were then visualized and image was documented using Gel documentation system (Molecular Imager Gel DOC ™ XR+ with ImageLab™ Software, BIO-RAD) under UV light. Band formations occur at 520 bp (DENG primer) or 575 bp (AV/AC primer) region of the lane corresponding to the ladder in case begomovirus was present (Appendix III).

### 3.6.3 Characterization of coat protein gene

Partial molecular characterization was undertaken by sequencing these coat protein amplicons at Agrigenome Pvt. Ltd. Kochi followed by sequence analysis of CP gene sequences using standard bioinformatics tools. The nucleotide sequences were compared by BLAST to sequence available at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.gov/. blast).

Table 2. Coat protein gene specific primers used for the detection of Begomoviruses in okra, tomato and pumpkin

| Primer | Sequences (5'-3') | Product size (bp) | References |
|---|---|---|---|
| AV494-F<br>AC1048-R | GCCHATRTAYAGRAA GCCMAGRAT<br>GGRTTDGARGCATGHGTACANGCC | 575 | Wyatt and Brown (1996) |
| DENG 541-F<br>DENG 540-R | TAATATTACCKGWKGVCCSC<br>TGGACYTTRCAWGGBCCTTCACA | 520 | Deng et al. (1992) |

Table 3. Temperature profile for the PCR amplification of coat protein gene of BYVMV, ToLCV and PYVMV

| Stage | Functions | Temperature (°C) | Time (min.) | No. of cycles |
|---|---|---|---|---|
| I | Initial denaturation | 92 | 1 | 1 |
| II | Denaturation<br>Primer Annealing<br>Primer extension | 92<br>58<br>72 | 1<br>1<br>1 | 35 |
| III | Final extension | 72 | 10 | 1 |

### 3.6.4 Full genome amplification of DNA-A of BYVMV, ToLCV and PYVMV

#### 3.6.4.1 Rolling Circle Amplification (RCA)

The full DNA isolated was put under RCA in order to get amplicons of the full genome. The circular DNA was amplified using RCA method (Haible *et al.*, 2006). Amplification of circular DNA was performed using 10-20 ng of total nucleic acids. The mixture was denatured for 3 min at 94°C and cooled down to room temperature. After cooling, 4 µl of pyrophosphatase (0.1 U $\mu l^{-1}$), 0.7 µl of phi 29 DNA polymerase (10U $\mu l^{-1}$) was added and kept for incubation for 18-20 h at 30°C and inactivated at 65°C for 10 min. 500 ng of amplified nucleic acids in 20 µl volume were digested by different restriction enzymes *viz.*, *Bam*HI, *Hind*III, *Kpn*I, *Sal*I and *Xba*I for 3 h at 37°C. The restriction products were run on agarose gel (2 %) following standard protocol (Sambrook and Russell, 2001).

#### 3.6.4.2 Restriction with different endonucleases

The restriction fragment analysis (RFA) was performed on the RCA products by digesting 2 µl (~600 ng DNA) of the RCA product using the restriction enzymes *Bam*HI, *Hind*III, *Xba*I, *Sal*I *and Kpn*I (GENETIX). This digestion was done to select those enzymes that could restrict at a single site in the genome of the begomovirus generating unit-size molecules, i.e. genome size of ~2.7 kb DNA fragment (DNA-A/DNA-B). The reaction was carried out at 37 °C for 2 h, followed by treatment for 20 min at 65 °C for enzyme inactivation. DNA fragments were separated on agarose gel (2 %) following standard protocols (Sambrook and Russell, 2001) and visualized by EtBr staining. Fragment sizes were estimated by comparison to known reference fragments of a molecular weight marker (Origin).

### 3.6.4.3 Development of primers for full genome amplification

Three sets of degenerative overlapping primers were designed based on multiple sequence alignment (MSA) of 22 begomovirus isolates that showed maximum similarity to the partial genome of the viruses under study. The MSA was done in Clustal W software.

### 3.6.4.4 PCR amplification of full genome of DNA-A

PCR amplification of the three begomoviruses *viz.*, BYVMV, ToLCV and PYVMV were done using the designed three sets of overlapping primers specific to the DNA-A of all the three viruses. The primers developed with their sequences and product sizes were mentioned in the Table 4.

The PCR amplification reactions were carried out in a 25 µl reaction mixture containing 10X PCR Taq buffer - 5.0 µl, 1.5 mM $MgCl_2$ - 1.5 ml, 10 mM dNTPs (dATP, dGTP, dCTP and dTTP) - 0.5 µl, 3.0 µl DNA, 0.3 µl (2.5 Units) Taq polymerase enzyme, forward and reverse primers (5 pM) 1 µl each (SIGMA-ALDRICH) and 12.7 µl sterile distilled water. The reaction mixture was run in a thermocycler (Veriti 96 well Thermal Cycler, Applied Biosystems) under the specified conditions (Table 5).

### 3.6.4.5 Agarose gel electrophoresis of PCR products

Agarose gel of 1.2 per cent was prepared in 0.5X TAE buffer containing 0.5 µg $ml^{-1}$ ethidium bromide (EtBr). 2 µl of 6X loading dye was mixed with 10 µl of PCR products, loaded and electrophoresis was performed at 75 V $cm^{-1}$ power supply with 0.5X TAE as electrophoresis buffer for about 1-2 h. The molecular standard used was a 1 kb DNA ladder (GeNei). The gels were then visualized and image was documented using Gel documentation system (Molecular Imager Gel DOC ™ XR+

Table 4. List of primers designed with their sequences and intended product (amplicon) size used for full genome amplification of BYVMV, ToLCV and PYVMV

| Primer | Primer Sequence (5'-3') | Product size (bp) |
|---|---|---|
| BIN 1 (F) | TATGKCGAAGCGWSCHSC | ~1000 |
| BIN 2 (R) | TGGGRVATDCACMARTGTKT | |
| BIN 3 (F) | TATATGGCHTGTACNCAYG | ~1300 |
| BIN 4 (R) | CRAACATHCAGGGAGCTAA | |
| BIN 5 (F) | GATCKDCCRTCGAYYTGRA | ~900 |
| BIN 6 (R) | TTTGTGACGCGGGCAATGG | |

Table 5. Temperature profile for PCR amplification of DNA-A of BYVMV, ToLCV and PYVMV

| Stage | Function | Temperature (°C) | Time (min.) | No. of cycles |
|---|---|---|---|---|
| I | Initial denaturation | 94 | 3 | 1 |
| II | Denaturation<br>Primer Annealing<br>Primer extension | 94<br>50<br>72 | 0.30<br>0.30<br>2 | 35 |
| III | Final extension | 72 | 10 | 1 |

with ImageLab™ Software) under UV light. Bands were formed at those regions of the lanes with product size corresponding to the designed primers (Table 4).

**3.6.4.6 Gel extraction of full genome**

The amplified DNA-A of the three viruses were then eluted using Gel extraction kit (GeneJET Gel Extraction Kit, Cat: #K0691, #K0692; Thermo Scientific). The protocol followed was as follows: Gel slice containing the DNA fragment was excised using a clean scalpel or razor blade. The cut was done as close to the DNA as possible so as to minimize the gel volume. The gel slice was then placed into a pre-weighed 1.5 ml tube and weighed. The weight of the gel slice was then recorded. Binding buffer was added in the ratio, 1:1 volume of Binding Buffer to the gel slice (volume: weight) (e.g., 100 µl of Binding Buffer was added for every 100 mg of agarose gel). The gel mixture was incubated at 50-60 °C for 10 min or until the gel slice was completely dissolved. The tube was mixed by inversion every minute to facilitate the melting process. The melting process was continued until the gel got completely dissolved in the binding buffer. The gel mixture was vortexed briefly before it was loaded on the column. The colour of the solution was checked. A yellow color indicated an optimal pH for DNA binding. Then 800 µl of the solubilized gel solution was transferred to the GeneJET purification column and centrifuged for 1 min. The flow-through was discarded the column was placed back into the same collection tube. 100 µl of the Binding Buffer was added to the GeneJET purification column and centrifuged for 1 min. Again, the flow-through was discarded and the column was placed back into the same collection tube. Further, 700 µl of Wash Buffer was added to the GeneJET purification column and centrifuged for 1 min. The flow-through was discarded as before and the column was placed back into the same collection tube. The empty GeneJET purification column was centrifuged for an additional 1 min to completely remove residual wash buffer. (This step was essential to avoid residual ethanol in the purified DNA solution to avoid

inhibition of downstream enzymatic reactions by ethanol). The GeneJET purification column was then transferred into a clean 1.5 ml microcentrifuge tube. 50 µl of Elution Buffer was added to the center of the purification column membrane and centrifuged for 1 min.). The GeneJET purification column was then discarded and the purified DNA was stored at -20 °C in a 1.5 ml eppendorf tube.

### 3.6.5 Characterization of full genome of DNA-A

The full genome characterization was undertaken by sequencing these amplicons at Agrigenome Pvt. Ltd. Kochi, and BLAST analysis was undertaken in NCBI site. Complete sequences of DNA- A of the three viruses were assembled using Contig Assembly Program on Bioedit software (Hall, 1999) and analyzed with BLAST (Altschul *et al.*, 1990). The full genome of BYVMV, ToLCNDV and SLCCNV were translated into protein sequences and the ORFs present in the sequences were determined by ORF finder (https://web.expasy.org/translate/) and the homology search of ORFs was performed using the tool BLASTp.

## 3.7 GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS

Genetic diversity and phylogenetic analysis of partial and full genome were done in MEGA 7 software. Phylogeny related studies were conducted in MEGA 7.0 (Kumar *et al.*, 2016) with neighbor joining algorithm (Tamura *et al.*, 2007). Bootstrapping for 500 replications was performed to determine the reliability of the tree. The genetic distance analysis of sequences was done using Genetic distance analysis application in MEGA 7.0 software (Kumar *et al.*, 2016).

## 3.8 DEVELOPING SPECIFIC PRIMERS FOR THE BEGOMOVIRUSES

The specific primers for BYVMV, ToLCV and PYVMV were developed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) after analyzing the ORF region of each viruses that showed maximum variation.

# Results

# 4. RESULTS

The begomoviruses infecting okra, tomato and pumpkin were always been a threat to the cultivation of these crops. Hence, this work was undertaken to study different modes of transmission, immunomolecular detection and genetic diversity of the three viruses *viz.,* BYVMV, ToLCV and PYVMV. Since a better understanding of the viruses involved would help to develop better management strategies. The results obtained in the study are presented in this chapter.

## 4.1 COLLECTION OF BEGOMOVIRUS INFECTED OKRA, TOMATO AND PUMPKIN

Collection of begomovirus infected okra, tomato and pumpkin plants were done in three taluks each of Thiruvananthapuram *viz.,* Neyyattinkara, Nedumangad and Chirayinkeezhu) and Kollam *viz.,* Kollam, Kottarakkara and Pathanapuram (Punalur) districts, Southern Kerala in the year 2017-2019 (Plate 1) (Table 6). Due to low incidence of pumpkin yellow vein disease in these two districts, the samples were also collected from Tavanur of Malappuram and Pilicode of Kasaragod districts. The samples were collected during February to November, 2018 by stratified random sampling method.

The infected crops in different locations were at different stages of crop and infection. Some crops were in their early stage of infection while the others were in their late stage with severe crop loss. Based on the number of plants infected in each field, the disease incidence was calculated for each location. In almost all locations cent per cent disease incidence was observed in bhendi yellow vein disease (BYVMD), tomato leaf curl disease (ToLCD) and pumpkin yellow vein disease (PYVMD).

Table 6. Begomovirus isolates of okra, tomato and pumpkin isolated from different locations of Kerala

| Sl. No. | District | Place of isolation | Isolate name | Crop variety |
|---|---|---|---|---|
| 1. | Thiruvananthapuram | COA, Vellayani | BYVMV Vellayani | Varsha Uphar |
| 2. | Thiruvananthapuram | Neyyattinkara | BYVMV Neyyattinkara | Salkeerthi |
| 3. | Thiruvananthapuram | Nedumangad | BYVMV Nedumangad | Salkeerthi |
| 4. | Thiruvananthapuram | Chirayinkeezhu | BYVMV Chirayinkeezhu | Salkeerthi |
| 5. | Kollam | Mukhathala | BYVMV Mukhathala | Kiran |
| 6. | Kollam | Kottarakkara | BYVMV Kottarakkara | Salkeerthi |
| 7. | Kollam | Punalur | BYVMV Punalur | Susthira |
| 8. | Thiruvananthapuram | COA,Vellayani | ToLCV Vellayani | Vellayani Vijay |
| 9. | Thiruvananthapuram | Neyyattinkara | ToLCV Neyyattinkara | Anagha |
| 10. | Thiruvananthapuram | Nedumangad | ToLCV Nedumangadu | Anagha |
| 11. | Thiruvananthapuram | Chirayinkeezhu | ToLCV Chirayinkeezhu | Anagha |
| 12. | Malappuram | Tavanur | ToLCV Tavanur | Anagha |
| 13. | Kollam | Mukhathala | ToLCV Mukhathala | Anagha |
| 14. | Kollam | Kottarakkara | ToLCV Kottarakkara | Anagha |
| 15. | Kollam | Punalur | ToLCV Punalur | Anagha |
| 16. | Kasaragod | Pilicode | PYVMV Kasaragod | Suvarna |
| 17. | Thiruvananthapuram | Pappanchani | PYVMV Pappanchani | Ambili |
| 18. | Malappuram | Tavanur | PYVMV Tavanur | Sooraj |
| 19. | Kollam | Mukhathala | PYVMV Mukhathala | Ambili |

In case of BYVMD in Thiruvananthapuram district, there was cent per cent D.I. in Vellayani, Neyyattinkara and Chirayinkeezhu with V.I. value 65.40, 70.00 and 69.30 respectively. While in Nedumangad the D.I. and V.I. values recorded were 60.00 and 44.00 respectively. In Kollam district, cent per cent disease incidence was observed in Mukhathala (Kollam taluk) with V.I. of 86.67. The D.I. in Kottarakkara taluk was recorded to be second highest in Kollam district with 94 per cent with V.I. of 66.00, while the least D.I. of 66 per cent was observed in Punalur (Pathanapuram taluk) with V.I. value 56.00 (Table 7). V.I. for BYVMD was calculated using score chart mentioned in Plate 3.

In Thiruvananthapuram district, out of the three taluks, ToLCD incidence was highest (100 %) in Vellayani, Neyyattinkara and Nedumangad with V.I. of 62.30, 65.20 and 80.00 respectively. Chirayinkeezhu taluk had least D.I. of 83 per cent with V.I. value 63.33. In Kollam district, cent per cent D.I. was observed in all the three locations *viz.* Mukhathala, Kottarakkara and Punalur with V.I. values 85.00, 60.00 and 71.00 respectively. In Tavanur of Malappuram district, cent per cent D.I. with V.I. of 68.00 was observed. Though ToLCD incidence was high in all the three districts and the highest D.I. was recorded in Mukhathala (Kollam taluk) (Table 7). V.I. for ToLCD was calculated using score chart mentioned in Plate 4. In this study the relation between whitefly population on V.I. was not studied.

In case of PYVMD incidence, three out of the four districts had cent per cent D.I. with V.I. values 72.80, 69.30 and 60.00 in Tavanur (Malappuram), Kasaragod and Pappanchani (Thiruvananthapuram) respectively. The least D.I., 90 per cent was observed in Mukhathala with V.I. value 64.00 (Table 7). V.I. for PYVMD was calculated using score chart mentioned in Plate 5.

Table 7. Disease incidence and vulnerability index of Begomoviruses infecting okra, tomato and pumpkin

| Sl. No. | Isolate name | Crop | Variety | D.I. (%) | V.I. |
|---|---|---|---|---|---|
| 1. | BYVMV Vellayani | Okra | Varsha Uphar | 100 | 65.40 |
| 2. | BYVMV Neyyattinkara | Okra | Salkeerthi | 100 | 70.00 |
| 3. | BYVMV Nedumangad | Okra | Salkeerthi | 60 | 44.00 |
| 4. | BYVMV Chirayinkeezhu | Okra | Salkeerthi | 100 | 69.30 |
| 5. | BYVMV Mukhathala | Okra | Kiran | 100 | 86.67 |
| 6. | BYVMV Kottarakkara | Okra | Salkeerthi | 66 | 56.00 |
| 7. | BYVMV Punalur | Okra | Susthira | 94 | 66.00 |
| 8. | ToLCV Vellayani | Tomato | Vellayani Vijay | 100 | 62.30 |
| 9. | ToLCV Neyyattinkara | Tomato | Anagha | 100 | 65.20 |
| 10. | ToLCV Nedumangad | Tomato | Anagha | 100 | 80.00 |
| 11. | ToLCV Chirayinkeezhu | Tomato | Anagha | 83 | 63.33 |
| 12. | ToLCV Tavanur | Tomato | Anagha | 100 | 68.00 |
| 13. | ToLCV Mukhathala | Tomato | Anagha | 100 | 85.00 |
| 14. | ToLCV Kottarakkara | Tomato | Anagha | 100 | 60.00 |
| 15. | ToLCV Punalur | Tomato | Anagha | 100 | 71.00 |
| 16. | PYVMV Kasaragod | Pumpkin | Suvarna | 100 | 69.30 |
| 17. | PYVMV Pappanchani | Pumpkin | Ambili | 100 | 60.00 |
| 18. | PYVMV Tavanur | Pumpkin | Sooraj | 100 | 72.80 |
| 19. | PYVMV Mukhathala | Pumpkin | Ambili | 90 | 64.00 |

D.I.: Disease incidence; V.I.: Vulnerability index

## 4.2 SYMPTOMATOLOGY

Symptoms expressed by *Bhendi yellow vein mosaic virus* (BYVMV) infected okra plants varied depending on the stage of infection. The early symptom of BYVMV infected okra plant observed was initial vein clearing which later on developed into typical vein clearing symptom where all the veins turned yellow in colour. In advanced stage of infection, the whole leaf turned chlorotic accompanied by blistering and distortion of leaf, reduced size of leaf, vein thickening, stunted growth, reduced flowering and fruiting, vein clearing of flower buds and malformed small sized fruits were observed. The symptoms of BYVMD observed from all the locations were similar to that mentioned above (Plate 6).

Symptoms observed in *Tomato leaf curl virus* (ToLCV) infected tomato plants included typical symptoms of leaf curling, distorted small sized leaves, stunted growth of plant; and reduced flowering and fruiting (Plate 7). Apart from the above-mentioned symptoms, in case ToLCV infected samples collected from Vellayani and Neyyattinkara of Thiruvananthapuram had additional symptoms of purplish discoloration of leaves, leaf cupping, yellowing and mottling of leaf lamina and virescence of flowers (Plate 8).

In this study the chances of mixed infection was not studied. The symptomatology of the three diseases were done with the assumption that there was no mixed infection in the three crops and all the symptoms expressed by the crops were as a result of infection by the corresponding viruses under study.

Symptomatology of *Pumpkin yellow vein mosaic* (PYVMV) infected pumpkin plants included initial vein clearing followed by mosaic of leaf lamina. In later stage of infection, the leaves became completely chlorotic with reduction in leaf size, stunted growth of plant, and reduction in flowering and fruiting and vein clearing on floral parts (Plate 9).

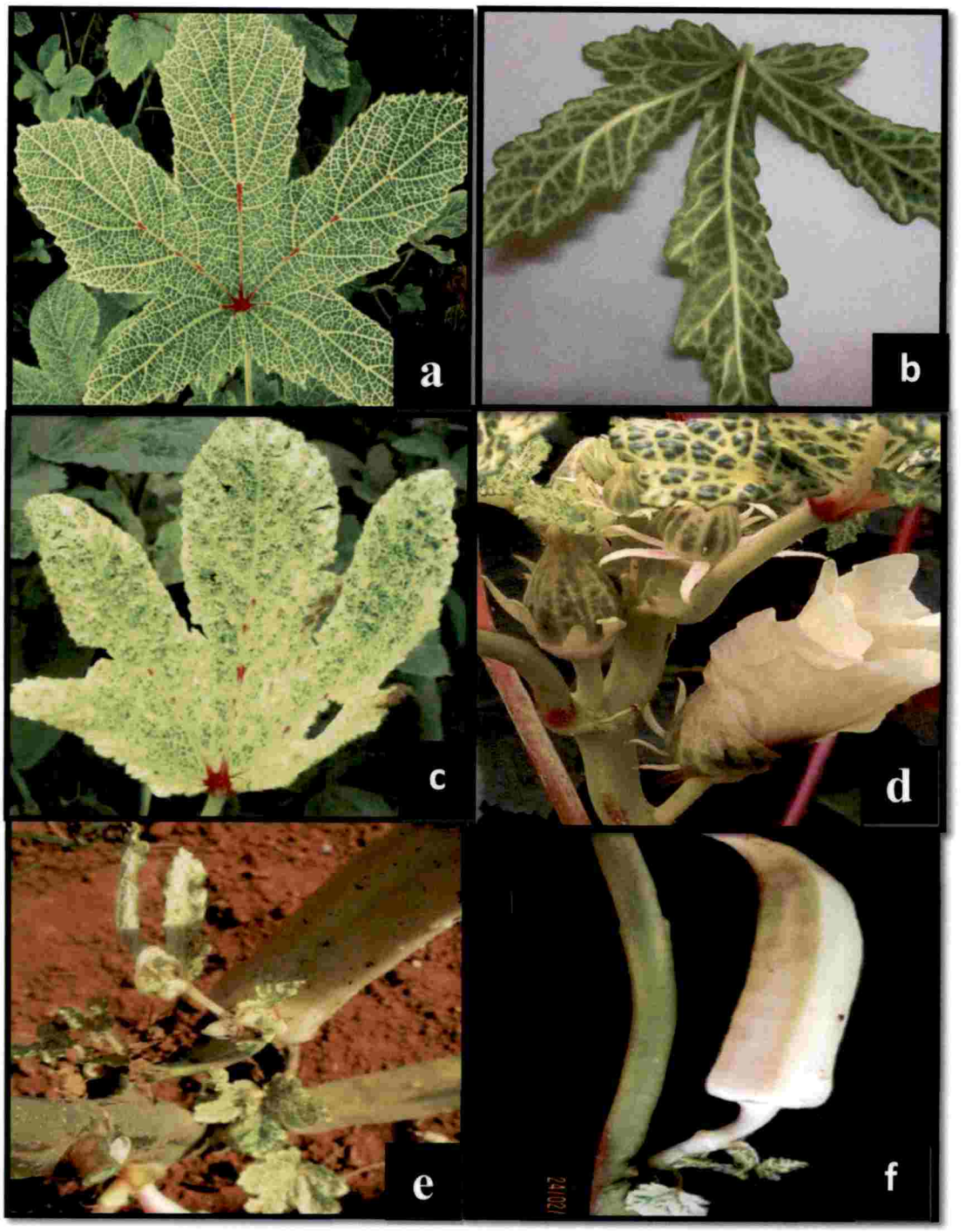



Plate 6. Different symptoms produced by BYVMV in okra plants. a) Vein clearing, b) vein thickening, c) crinkling of leaves, d) bleached appearance of fruits, e) distorted and small sized leaves with slight curling and f) vein clearing on flower buds.

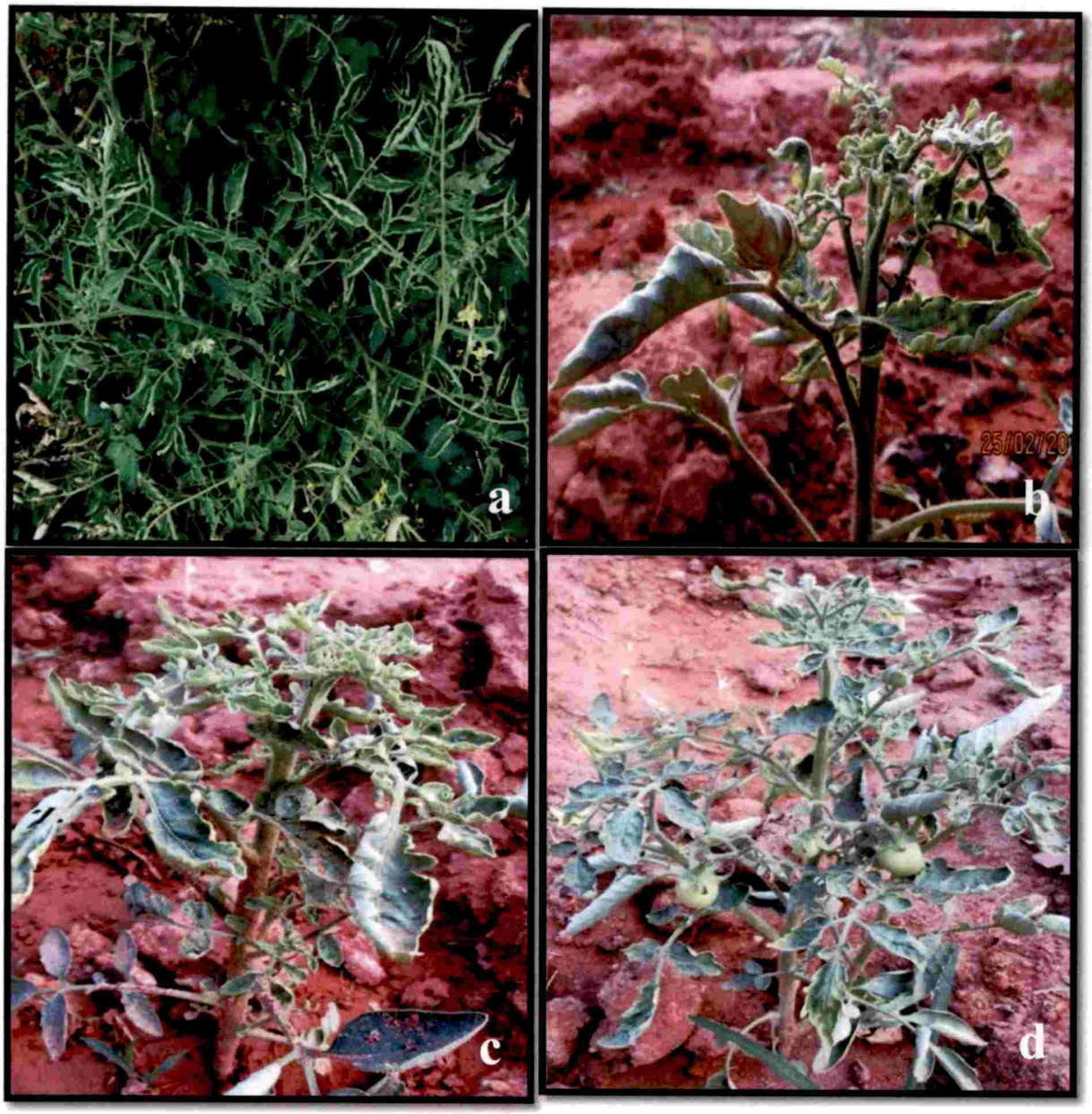



Plate 7. Different symptoms produced by ToLCV in tomato plants. (a) Leaf curling, (b) distorted small sized leaves, (c) distorted small sized leaves and (d) reduced fruit bearing and small sized fruits.

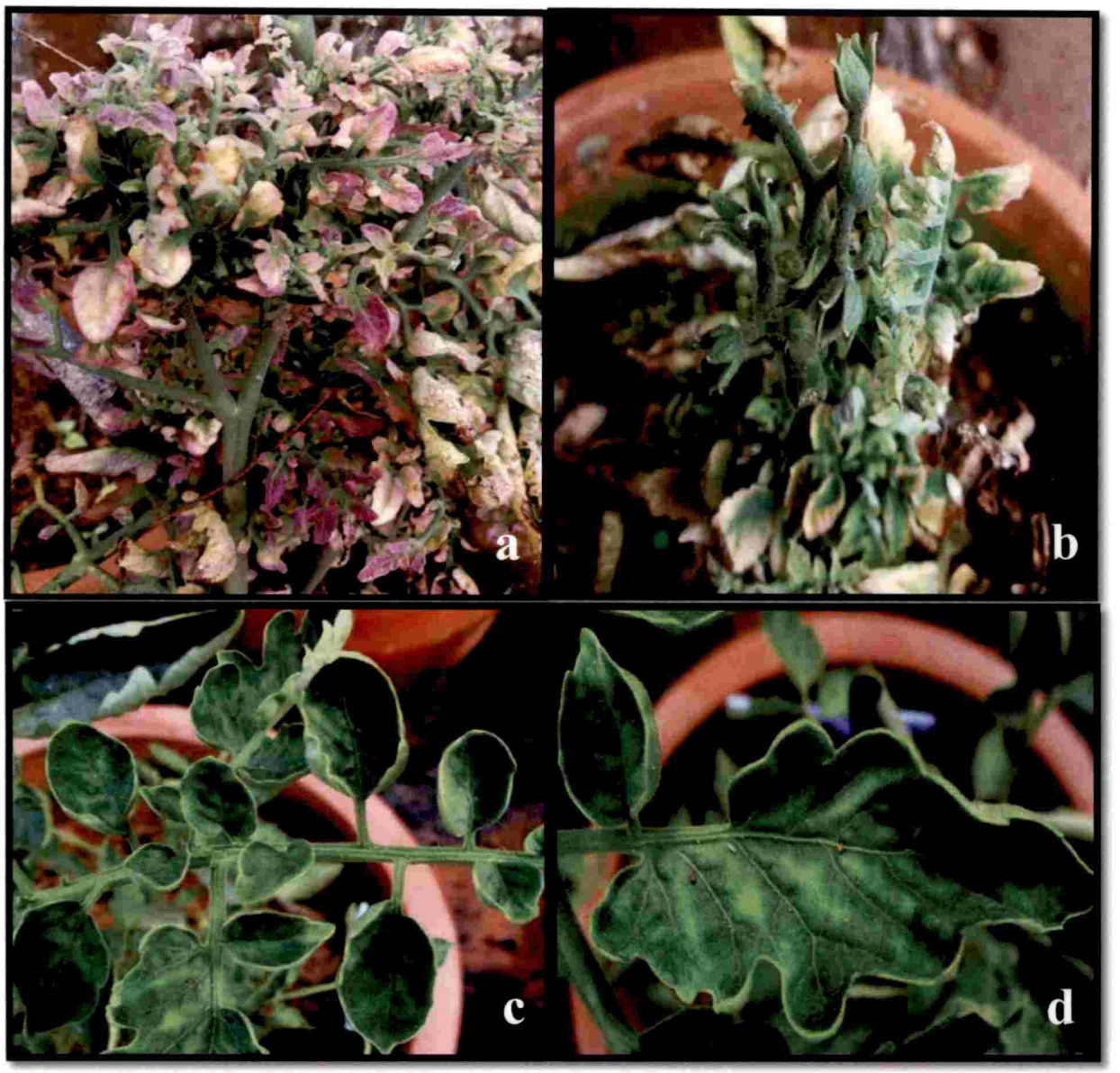



Plate 8. Different symptoms produced by ToLCV in tomato plants. (a) Purplish discoloration of leaves, (b) virescence of flowers, (c) leaf cupping and (d) mottling and yellowing of leaf lamina.

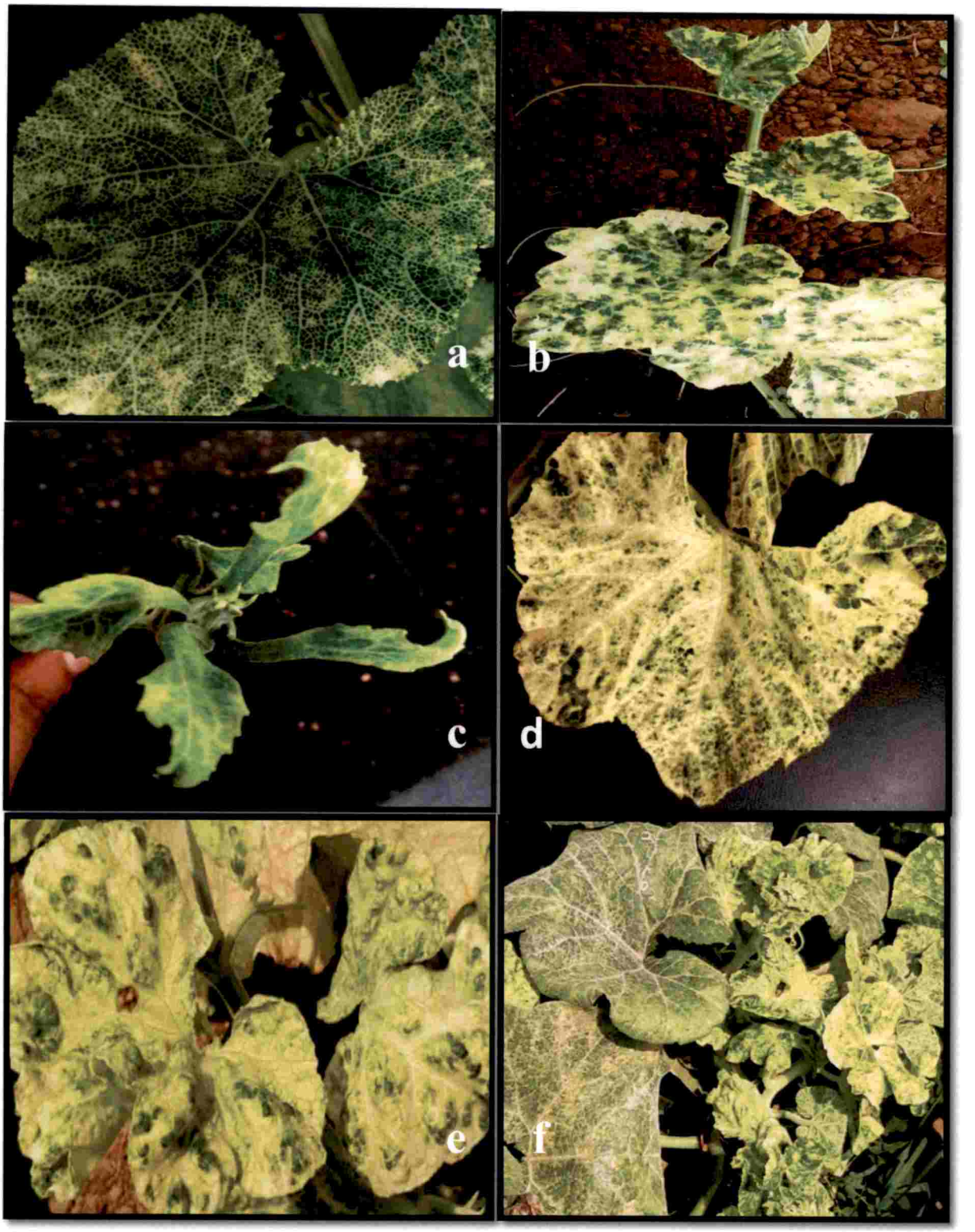



Plate 9. Different symptoms produced by PYVMD in pumpkin plants. (a) Vein clearing, b) mosaic symptom, c) yellowing of veins in flower (sepals), d) complete chlorosis of leaf and veins, e) crinkling and blistering of leaf and f) reduced leaf size of pumpkin leaves.

Days taken for symptom development in case of each of the three diseases were as mentioned in the Table 8. First symptom developed after three weeks of inoculation of the virus using whiteflies in the respective crops.

## 4.3. TRANSMISSION STUDIES

Transmission studies were done to understand different modes of transmission of BYVMV, ToLCV and PYVMV virus.

### 4.3.1 Insect transmission

Insect transmission studies were conducted using whiteflies (*B. tabaci* Genn.) (Plate 2). In case of BYVMV transmission study in okra seedlings, cent per cent the insect transmission was observed in variety Varsha Uphar (Plate 10). Similarly, in tomato seedlings also cent per cent ToLCV transmission was observed in variety Vellayani Vijai (Plate 11). In pumpkin seedlings only 66.60 per cent insect transmission of PYVMV was observed in variety Ambili (Plate 12) (Table 9).

### 4.3.2 Graft transmission

Graft transmission study of BYVMV, ToLCV and PYVMV on okra, tomato and pumpkin seedlings respectively was done using wedge graft method. In case of both BYVMV and ToLCV, cent per cent graft transmission of the virus were noticed while in case of pumpkin seedling, only 42.80 per cent graft transmission was observed (Table 10). The initial slight vein clearing appeared in grafted okra seedling 8 days after grafting (DAG) while 14 DAG, typical vein clearing symptom was expressed (Plate 13). In ToLCV transmission, slight leaf curling appeared 10 DAG in tomato seedlings and symptom further developed to complete leaf curling with distortion of leaves after 22 days of grafting (Plate 14). Whereas in PYVMV, the grafted seedlings expressed typical vein clearing after 10 days of grafting (Plate 15).

Table 8. Symptoms expressed by begomoviruses infection in okra, tomato and pumpkin

| Sl. No. | Crop | Disease | Symptoms developed | DTSD* |
|---|---|---|---|---|
| 1. | Okra | BYVMD | Vein clearing, vein thickening, mosaic, chlorosis of leaf, crinkling and puckering of leaves, slight leaf curling, stunted growth and reduced fruit set, chlorosis and hardening of fruit | 22 |
| 2. | Tomato | ToLCD | Leaf curling, leaf cupping, mottling and yellowing of leaves, purplish discoloration of leaves, virescence of floral parts, stunted growth, reduction in fruit set and size of leaves | 20 |
| 3. | Pumpkin | PYVMD | Vein clearing, crinkling and puckering of leaves, mosaic, chlorosis, stunted growth | 23 |

*DTSD - Days taken for symptom development

Table 9. Whitefly transmission of BYVMV, ToLCV and PYVMV in okra, tomato and pumpkin

| Sl. No. | Begomovirus | Number of seedlings inoculated | Number of seedlings infected | Days taken for symptom development | Percentage insect transmission (%) | V.I. |
|---|---|---|---|---|---|---|
| 1. | BYVMV | 9 | 9 | 20 | 100 | 58 |
| 2. | PYVMV | 6 | 4 | 20 | 66.6 | 62 |
| 3. | ToLCV | 8 | 8 | 20 | 100 | 50 |

Table 10. Graft transmission of BYVMV, ToLCV and PYVMV in okra, tomato and pumpkin

| Sl. No. | Crop | Begomovirus | Number of seedlings grafted | Number of seedlings infected | Days taken for symptom development | Percentage graft transmission (%) | V.I. |
|---|---|---|---|---|---|---|---|
| 1. | Okra | BYVMV | 15 | 15 | 7-14 | 100 | 70.7 |
| 2. | Pumpkin | PYVMV | 7 | 3 | 10-12 | 42.8 | 54.0 |
| 3. | Tomato | ToLCV | 10 | 10 | 18-20 | 100 | 66.6 |

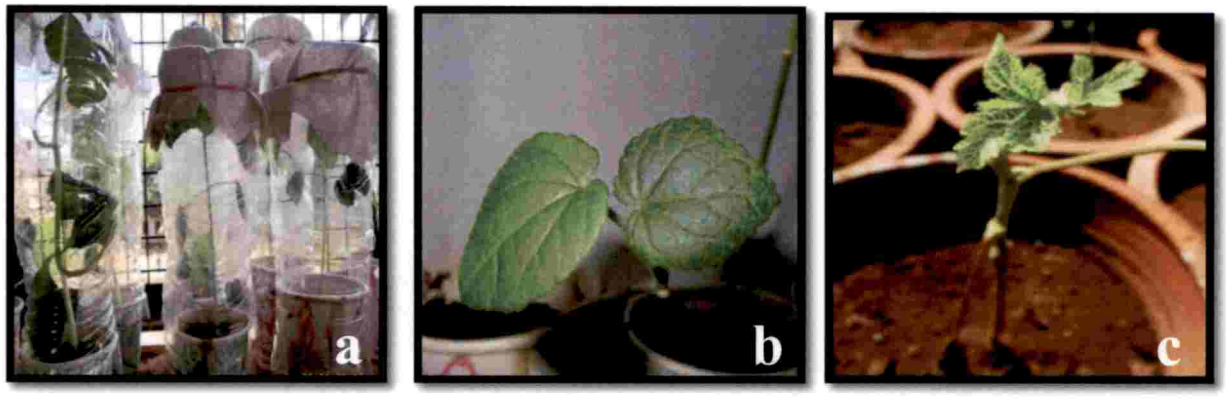



Plate 10. a) Seedlings raised in insect proof cages for whitefly transmission of BYVMV b) Okra plant prior to the inoculation c) Okra plant after 22 days of the inoculation showing vein clearing


Plate 11. a) Healthy tomato seedlings raised in insect proof cages b) Tomato seedlings after 20 days of the inoculation showing light curling of leaf c) Tomato seedlings after 25 days of the inoculation, more leaves expressing leaf curling symptom.

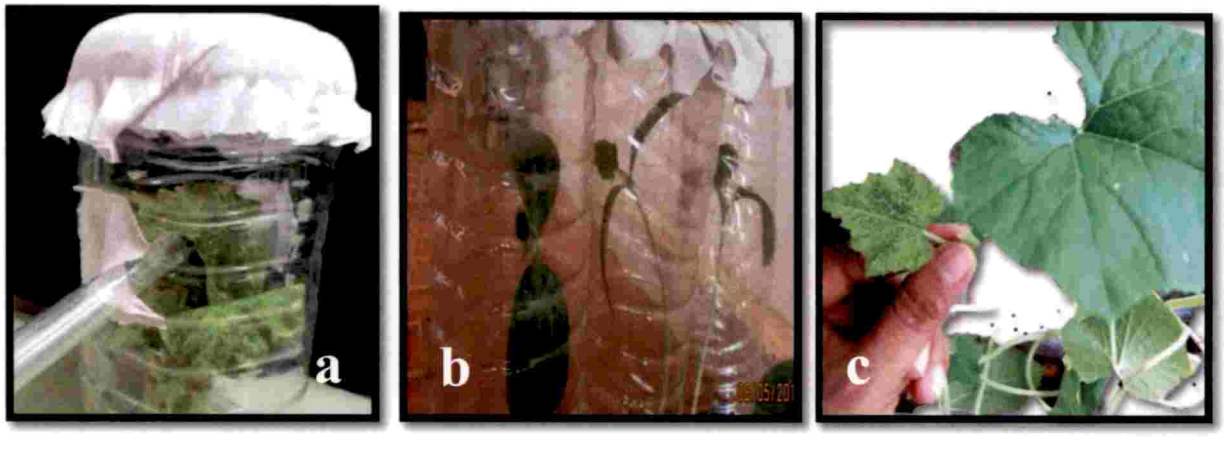



Plate 12. a) Whiteflies being released on infected pumpkin plant sample for virus inoculation, b) healthy pumpkin seedlings raised inside insect proof cage and c) appearance of vein clearing symptom 23 days after inoculation using whiteflies

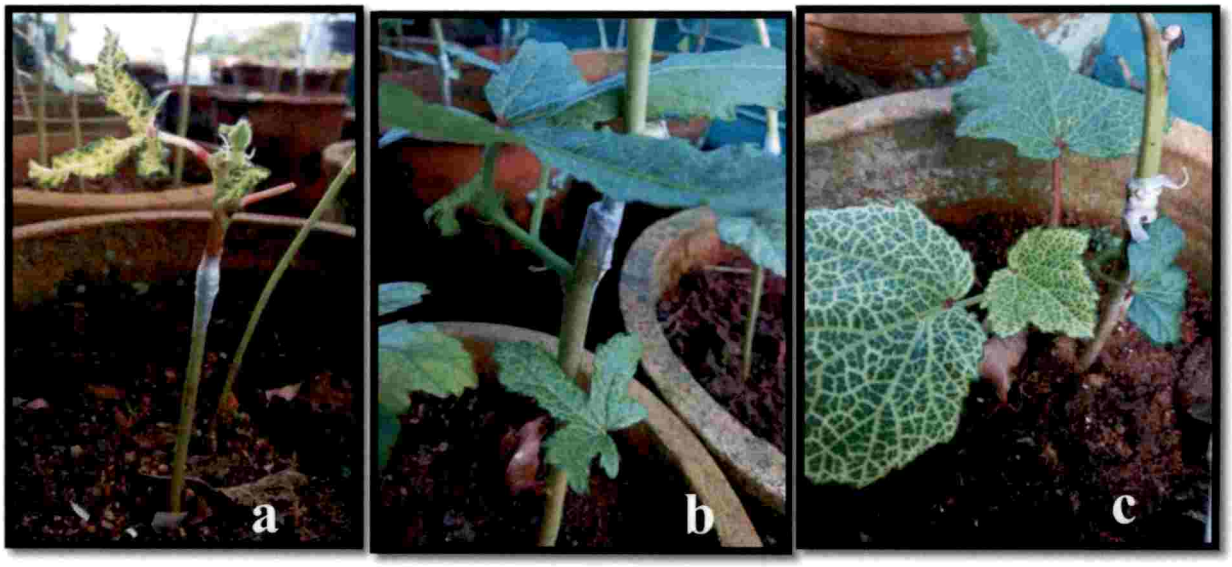



Plate 13. Graft transmission of BYVMV in okra seedlings by wedge grafting. a) Grafted okra seedlings, b) Okra plants after 8 days of the grafting expressing slight vein clearing of leaf and c) Okra plant after 14 days of the grafting expressing typical vein clearing

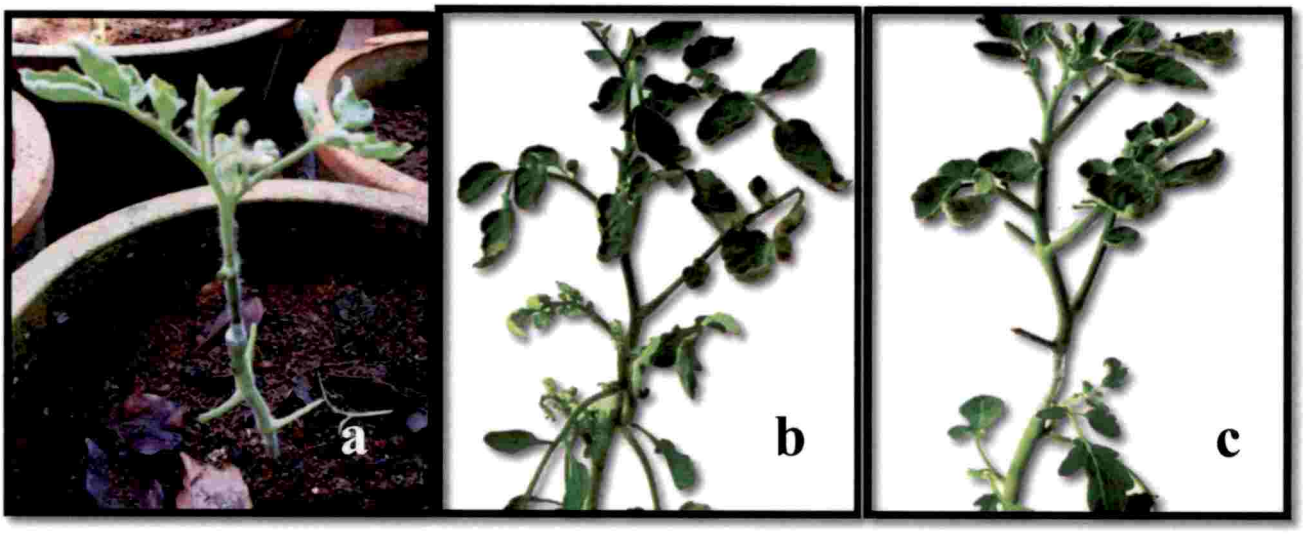



Plate 14. Graft transmission of ToLCV by wedge grafting. a) Grafted tomato seedling, b) 10 days old grafted plant with slight curling of leaf, c) 22 days old grafted plant with typical leaf curling symptom.


Plate 15. Graft transmission of PYVMV in pumpkin seedlings by wedge grafting. a) and b) Pumpkin seedlings after grafting and c) Pumpkin seedling showing vein clearing symptom after 10 days of the grafting


Plate 16. Seed transmission of BYVMV. a) and b) 3 months old okra plant raised from seeds of the infected plants expressing no BYVMD symptom

### 4.3.3 Seed transmission

Seed transmission study was done using seeds collected from the begomovirus infected okra, tomato and pumpkin plants. None of the seedlings raised under insect proof condition did express any kind of symptom even after three months of incubation period (Table 11) (Plate 16-18).

## 4.4 CROSS-INFECTION STUDY

Cross-infection study of the three begomoviruses in the okra, tomato and pumpkin seedlings were done using whiteflies but none of the seedlings expressed any kind of symptom development even after one-month of incubation period under insect proof condition (Table 12) (Plate 19-21).

## 4.5 IMMUNO-DETECTION

### 4.5.1 Immuno-detection by Enzyme linked immunosorbent assay (ELISA)

Immuno-detection was carried out using DAS-ELISA using antisera specific to ToLCNDV and TAS-ELISA using antisera specific to viruses namely *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) as they were the only antisera available to test the presence of the respective viruses in the samples collected. The antisera were procured from DSMZ, Germany. The absorbance was read at 405 nm in an ELISA reader (Microplate Reader 680, BIORAD) (Plate 22). The ratio of absorbance value of infected samples to healthy samples was found to be higher than 2 in case of all the infected leaf samples, confirming presence of begomoviruses in tested samples. Hence the presence of the begomoviruses in all samples were detected using the above antisera as shown in Table 13. While ELISA detection in seeds collected from infected plants using ToLCNDV antiserum was tested negative since the ratio of absorbance value of diseased to healthy samples were less than 2, as shown in Table 14.

Table 11. Seed transmission of Begomoviruses using seeds from infected plants

| Sl. No. | Infected Crop | Begomovirus | Number of seedlings raised | Days taken for symptom development | Percentage seed transmission | V.I. |
|---|---|---|---|---|---|---|
| 1. | Okra | BYVMV | 10 | No symptom development | | |
| 2. | Tomato | ToLCV | 10 | | | |
| 3. | Pumpkin | PYVMV | 10 | | | |

Table 12. Cross-infection of the Begomoviruses in okra, tomato and pumpkin seedlings using whiteflies

| Sl. No. | Begomovirus | Crops inoculated | Number of seedlings | Days taken for symptom development | Percentage cross-infection | V.I. |
|---|---|---|---|---|---|---|
| 1. | BYVMV | Pumpkin | 10 | No symptom development | | |
| | | Tomato | 10 | | | |
| 2. | ToLCV | Okra | 10 | | | |
| | | Tomato | 10 | | | |
| 3. | PYVMV | Okra | 10 | | | |
| | | Pumpkin | 10 | | | |

Table 13. Reaction of okra, tomato and pumpkin leaf samples infected by begomovirus in DAS/TAS-ELISA

| Sl. No. | Antiserum used | Begomovirus isolates | Absorbance value at 405 nm (mean of 3 values) | | Ratio of absorbance value at 405 nm | Remarks |
|---|---|---|---|---|---|---|
| | | | Diseased | Healthy | | |
| 1. | ACMV antiserum | BYVMV Vellayani | 0.196 | 0.061 | 3.2 | Positive |
| 2. | ToLCNDV antiserum | BYVMV Neyyattinkara | 0.250 | 0.110 | 2.2 | Positive |
| 3. | ToLCNDV antiserum | BYVMV Nedumangad | 0.007 | 0.003 | 2.3 | Positive |
| 4. | ACMV antiserum | BYVMV Chirayinkeezhu | 0.331 | 0.073 | 3.4 | Positive |
| 5. | SLCMV antiserum | BYVMV Mukhathala | 0.024 | 0.009 | 2.6 | Positive |
| 6. | SLCMV antiserum | BYVMV Kottarakkara | 0.018 | 0.006 | 2.5 | Positive |
| 7. | SLCMV antiserum | BYVMV Punalur | 0.032 | 0.012 | 2.7 | Positive |
| 8. | ToLCNDV antiserum | ToLCV Vellayani | 0.321 | 0.094 | 3.4 | Positive |
| 9. | ToLCNDV antiserum | ToLCV Vellayani | 0.611 | 0.190 | 3.2 | Positive |
| 10. | ToLCNDV antiserum | ToLCV Nedumangad | 0.052 | 0.017 | 3.0 | Positive |
| 11. | ToLCNDV antiserum | ToLCV Chirayinkeezhu | 0.078 | 0.027 | 2.8 | Positive |
| 12. | ACMV antiserum | ToLCV Tavanur | 0.083 | 0.029 | 2.8 | Positive |
| 13. | ACMV antiserum | ToLCV Mukhathala | 0.053 | 0.021 | 2.5 | Positive |
| 14. | ACMV antisera | ToLCV Kottarakkara | 0.015 | 0.006 | 2.5 | Positive |
| 15. | SLCMV antiserum | ToLCV Punalur | 0.014 | 0.006 | 2.3 | Positive |
| 16. | ToLCNDV antiserum | PYVMV Kasaragod | 0.130 | 0.040 | 3.5 | Positive |
| 17. | ToLCNDV antiserum | PYVMV Pappanchani | 0.073 | 0.022 | 3.2 | Positive |
| 18. | ToLCNDV antiserum | PYVMV Tavanur | 0.468 | 0.123 | 3.8 | Positive |
| 19. | ToLCNDV antiserum | PYVMV Mukhathala | 0.296 | 0.092 | 3.2 | Positive |

Table 14. Reaction of okra, tomato and pumpkin seed samples from Begomovirus infected plants in DAS-ELISA using polyclonal antibody of ToLCNDV

| Sl. No. | Crop | Begomovirus isolates | Absorbance value at 405 nm | | Ratio of absorbance value at 405 nm | Remarks |
|---|---|---|---|---|---|---|
| | | | Diseased | Healthy | | |
| 1. | Tomato | ToLCV Vellayani | 0.023 | 0.015 | 1.5 | Negative |
| 2. | Okra | BYVMV Vellayani | 0.019 | 0.016 | 1.1 | |
| 3. | Pumpkin | PYVMV Mukhathala | 0.015 | 0.015 | 1.0 | |


Plate 16. Seed transmission of BYVMV. a) and b) 3 months old okra plant raised from seeds of the infected plants expressing no BYVMD symptom


Plate 17. Seed transmission of ToLCV. a) 3-4 week-old seedlings of tomato raised from seeds of the infected plants b) 2 months old seedlings of tomato without the symptoms.


Plate 18. Seed transmission of PYVMV. a) and b) 2 months old seedlings of pumpkin raised from the seeds of the infected plants showing no symptoms.


Plate 19. Cross infection study of BYVMV using whiteflies. a) Tomato seedling 3 week after inoculation and b) Pumpkin seedling 2 weeks after inoculation without the symptoms

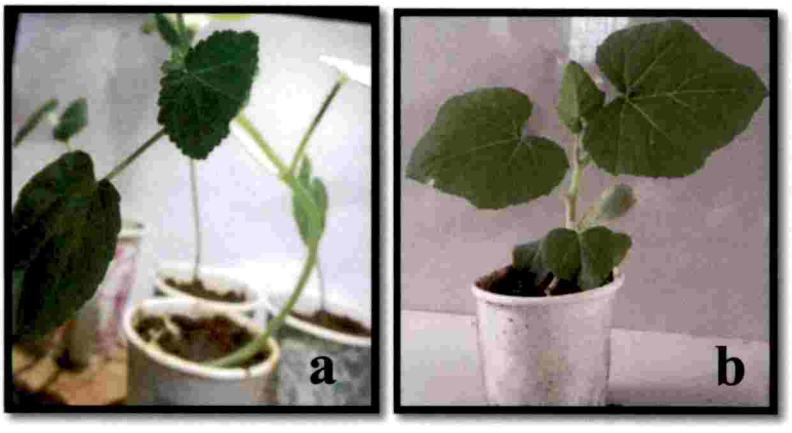



Plate 20. Cross infection study of ToLCV using whiteflies. a) Okra seedling 3 week after inoculation and b) Pumpkin seedling 3 weeks after inoculation without the symptoms


Plate 21. Cross infection study of PYVMV using whiteflies. a) Tomato seedling and b) Okra seedling two week after inoculation without the symptoms

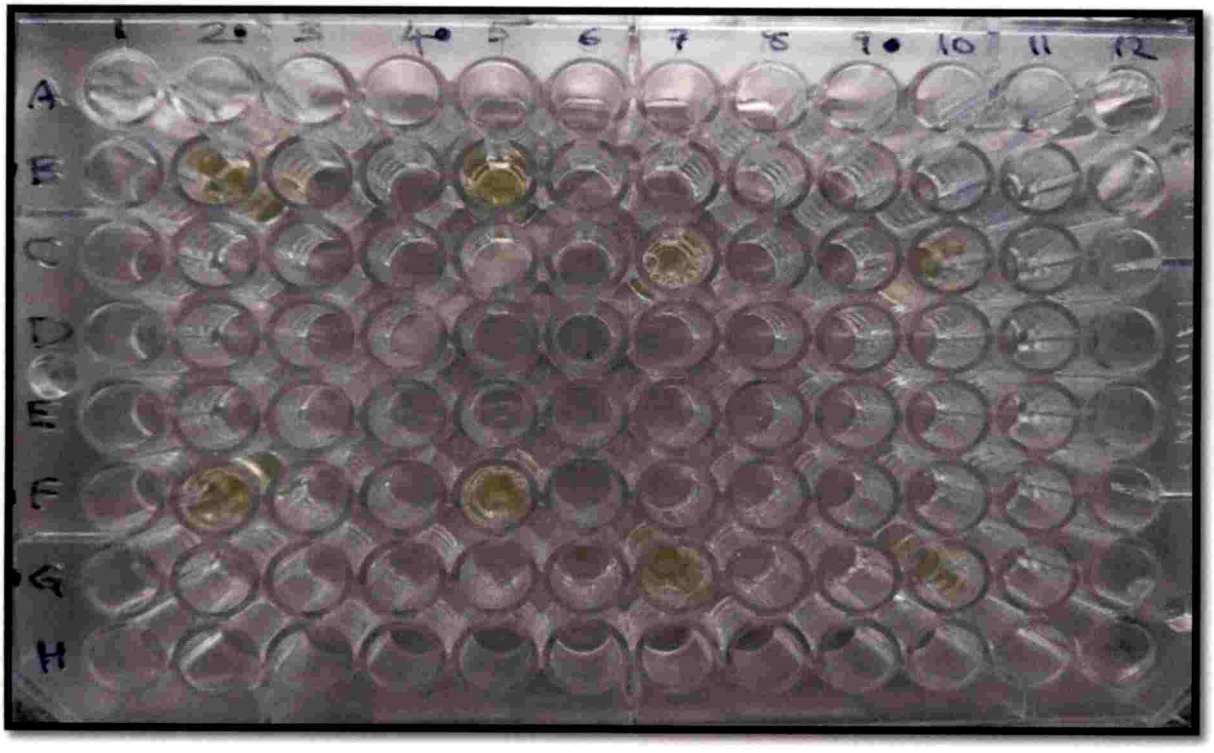



Plate 22. Colour development in ELISA plate by interaction of the Begomovirus infected samples with the polyclonal antiserum specific to ToLCNDV. B2 and B5- ToLCV Vellayani sample, F2 and F5-ToLCV Neyyattinkara samples, C7 and C10- Healthy sample and G7 and G10- Blank.

### 4.5.2 Immuno-detection by Dot immunobinding assay (DIBA)

Immuno-detection using DIBA was done on the begomoviruses infected samples using antiserum specific to ToLCNDV and all the samples confirmed the begomoviruses infection since color developed was more intense in the NCM membrane spotted with infected samples compared to healthy sample (Plate 23). The gel documentation value of NCM membrane further confirmed virus infection in the samples tested, since the gel doc readings were higher for disease samples compared to healthy sample (Table 15).

## 4.6 MOLECULAR DIAGNOSIS AND CHARACTERIZATION

### 4.6.1 Isolation of genomic DNA

The total nucleic acids from plant samples were extracted using DNeasy plant mini kit (QIAGEN: Cat. No. 69104).

The quantity and quality of the isolated virus DNA were analyzed in Bio-Spectrophotometer as shown in Table 16. The quality of extracted DNA (ratio of 260 nm to 280 nm) ranged from 1.11 to 1.50. Among the DNA samples, Vellayani isolate of ToLCV had high quality DNA (1.50) with DNA quantity 369.6 ng $\mu l^{-1}$ while Mukhathala isolate of ToLCV was found to have least quality (1.11) with DNA quantity 1138.5 ng $\mu l^{-1}$.

### 4.6.2 Amplification of coat protein gene of BYVMV, ToLCV and PYVMV

Molecular diagnosis was carried out with the virus infected okra, tomato and pumpkin plants. DNA was isolated and subjected to PCR with two universal primers specific to coat protein of begomoviruses. The begomoviruses isolated from tender seeds collected from infected okra, tomato and pumpkin did amplify the product size ~575bp with AV/AC and 520 bp with the DENG primer though mature seeds from all these crops did not yield amplicons of required size (Table 17 and 18; Plate 24 and

Table 15. Gel doc analysis report of DIBA of BYVMV, PYVMV and ToLCV infected leaf samples using antiserum of ToLCNDV

| Sl. No. | Begomovirus isolates | Label | Type | Volume (Int) | Min. Value (Int) | Max. Value (Int) | Mean Value (Int) | Std. Dev. | Area ($mm^2$) |
|---|---|---|---|---|---|---|---|---|---|
| 1. | BYVMV Neyyattinkara | U1 | H | 6,475,990 | 1,074 | 2,333 | 1,399.1 | 126.2 | 22.5 |
| | | U2 | D | 6,762,082 | 1,201 | 2,593 | 1,573.0 | 149.1 | 19.2 |
| | | B1 | Blank | 6,099,717 | 1,062 | 3,266 | 1,325.7 | 220.2 | 21.4 |
| 2. | BYVMV Chirayinkeezhu | U1 | H | 7,050,741 | 1,123 | 2,074 | 1,532.4 | 151.9 | 21.4 |
| | | U2 | D | 7,342,953 | 1,117 | 2,803 | 1,595.9 | 213.6 | 21.4 |
| | | B1 | Blank | 4,802,275 | 1,096 | 2,320 | 1,306.0 | 99.5 | 17.1 |
| 3. | BYVMV Mukhathala | U1 | D | 6,712,402 | 1,226 | 2,368 | 1,720.7 | 170.1 | 18.2 |
| | | U2 | H | 5,333,324 | 1,144 | 2,072 | 1,637.5 | 153.8 | 15.2 |
| | | B1 | Blank | 5,096,792 | 1,134 | 2,170 | 1,386.1 | 146.9 | 17.1 |
| 4. | PYVMV Mukhathala | U1 | D | 7,879,994 | 926 | 2,757 | 1,284.0 | 145.3 | 28.6 |
| | | U2 | H | 5,672,867 | 996 | 2,028 | 1,305.6 | 140.1 | 20.2 |
| | | B1 | Blank | 6,510,896 | 1,117 | 2,455 | 1,347.2 | 128.9 | 22.5 |
| 5. | ToLCV Neyyattinkara | U1 | D | 7,070,315 | 1,312 | 2,524 | 1,717.3 | 144.5 | 19.2 |
| | | U2 | H | 4,037,590 | 1,112 | 2,500 | 1,399.5 | 120.7 | 13.4 |
| | | B1 | Blank | 2,674,408 | 1,137 | 2,113 | 1,331. 2 | 70.4 | 9.4 |
| 6. | ToLCV Nedumangadu | U1 | D | 8,163,845 | 1,465 | 2,940 | 1,878.9 | 174.5 | 20.2 |
| | | U2 | H | 4,625,664 | 1,127 | 3,008 | 1,509.2 | 195.4 | 14.3 |
| | | B1 | Blank | 3,386,278 | 1,093 | 2745 1 | 1,261.2 | 118.1 | 12.5 |
| 7. | ToLCV Chirayinkee-zh u | U1 | D | 7,481,195 | 1,144 | 1,968 | 1,547.9 | 133.9 | 22.5 |
| | | U2 | H | 6,060,222 | 1,089 | 1,863 | 1,394. 8 | 98.7 | 20.2 |
| | | B1 | Blank | 5,618,612 | 1,098 | 1,737 | 1,293.1 | 54.1 | 20.2 |

D: Disease sample; H: Healthy sample

Table 16. Biospectrophotometer analysis of quality and quantity of DNA of Begomoviruses *viz.* BYVMV, ToLCV and PYVMV

| Sl. No. | Isolate name | OD at 260 nm | OD at 280 nm | 260/280 value | Quantity of DNA (ng µl-1) |
|---|---|---|---|---|---|
| 1. | BYVMV Vellayani | 0.169 | 0.130 | 1.30 | 557.7 |
| 2. | BYVMV Neyyattinkara | 0.208 | 0.170 | 1.22 | 686.4 |
| 3. | BYVMV Nedumangad | 0.152 | 0.127 | 1.18 | 501.6 |
| 4. | BYVMV Chirayinkeezhu | 0.258 | 0.226 | 1.14 | 851.4 |
| 5. | BYVMV Mukhathala | 0.222 | 0.168 | 1.32 | 732.6 |
| 6. | BYVMV Kottarakkara | 0.364 | 0.291 | 1.25 | 1201.2 |
| 7. | BYVMV Punalur | 0.163 | 0.119 | 1.36 | 537.9 |
| 8. | ToLCV Vellayani | 0.112 | 0.074 | 1.50 | 369.6 |
| 9. | ToLCV Neyyattinkara | 0.521 | 0.374 | 1.39 | 1719.3 |
| 10. | ToLCV Nedumangad | 0.237 | 0.188 | 1.26 | 782.1 |
| 11. | ToLCV Chirayinkeezhu | 0.161 | 0.134 | 1.20 | 531.3 |
| 12. | ToLCV Tavanur | 0.466 | 0.330 | 1.41 | 1537.8 |
| 13. | ToLCV Mukhathala | 0.345 | 0.310 | 1.11 | 1138.5 |
| 14. | ToLCV Kottarakkara | 0.174 | 0.146 | 1.19 | 574.2 |
| 15. | ToLCV Punalur | 0.182 | 0.131 | 1.38 | 600.6 |
| 16. | PYVMV Kasaragod | 0.155 | 0.123 | 1.26 | 511.5 |
| 17. | PYVMV Pappanchani | 0.193 | 0.159 | 1.21 | 636.9 |
| 18. | PYVMV Tavanur | 0.228 | 0.180 | 1.26 | 752.4 |
| 19. | PYVMV Mukhathala | 0.352 | 0266 | 1.32 | 1161.6 |

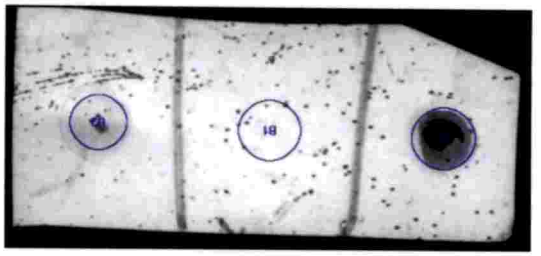
OD: Optical density value

Table 17. PCR amplification of Begomovirus isolates from the tender seeds (immature seeds) of the infected okra, tomato and pumpkin plants using AV/AC and DENG primers

| Sl. No. | Crop | Begomovirus isolates | Primers | |
|---|---|---|---|---|
| | | | AV/AC | DENG |
| 1. | Okra | BYVMV Vellayani | + | + |
| 2. | Tomato | ToLCV Vellayani | + | + |
| 3. | Pumpkin | PYVMV Mukhathala | + | + |

Table 18. PCR amplification of Begomovirus isolates from the mature dried seeds of the infected okra, tomato and pumpkin plants using AV/AC and DENG primers

| Sl. No. | Crop | Isolate | Primers | |
|---|---|---|---|---|
| | | | AV/AC | DENG |
| 1. | Okra | BYVMV Vellayani | No amplification | |
| 2. | Tomato | ToLCV Vellayani | | |
| 3. | Pumpkin | PYVMV Mukhathala | | |



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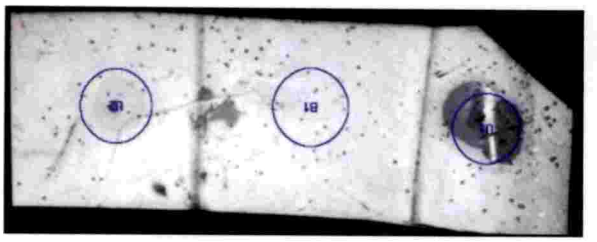

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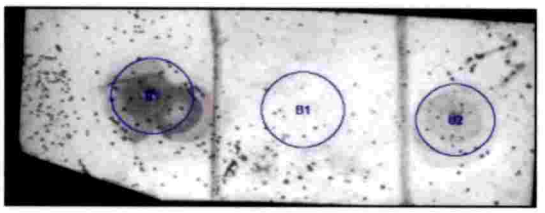

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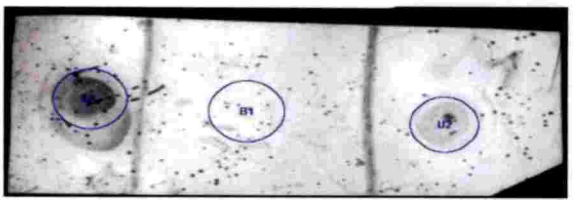

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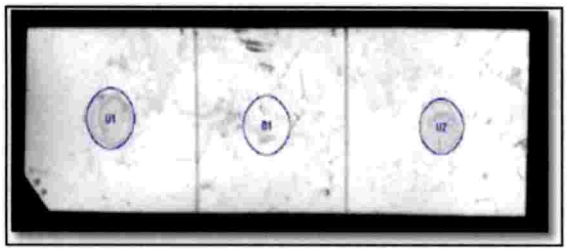

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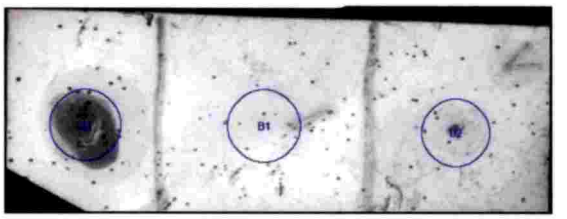

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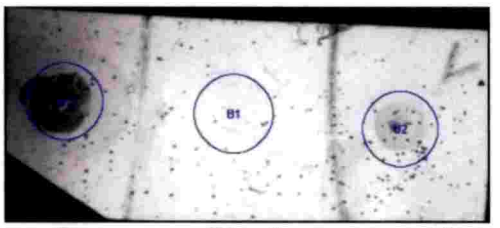

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Plate 23. Colour development on nitrocellulose membrane by DIBA analysis of the Begomovirus infected samples using polyclonal antiserum of ToLCNDV. a) BYVMV Neyyattinkara, (b) BYVMV Chirayinkeezhu, c) BYVMV Mukhathala, (d) PYVMV Mukhathala, e) ToLCV Nedumangad, f) ToLCV Chirayinkeezhu and (g) ToLCV Neyyattinkara.

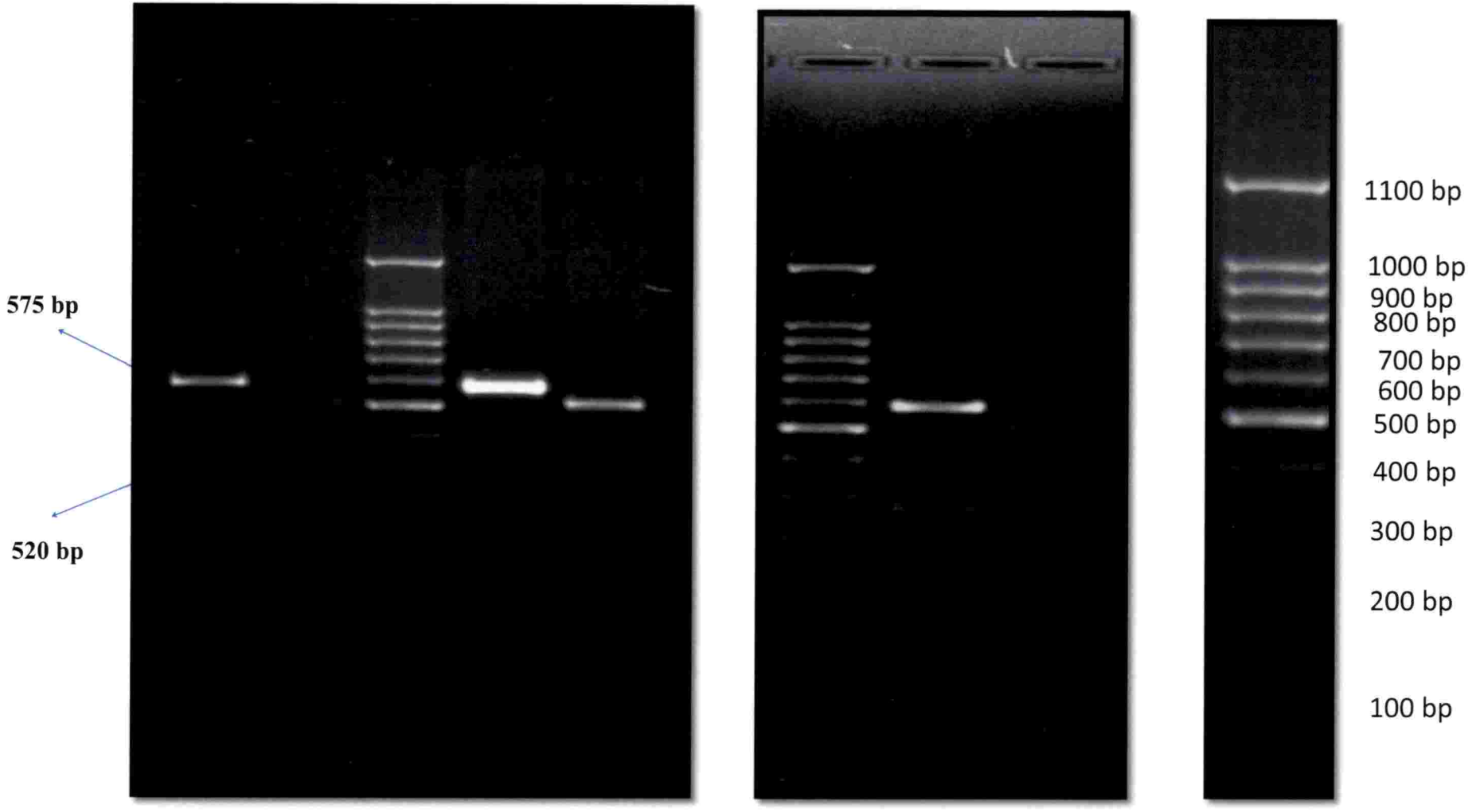


Plate 24. Amplification of coat protein gene of Begomoviruses isolated from tender seeds of the infected tomato, okra and pumpkin using AV/AC (575 bp) and DENG primer (520 bp). (a) M-100 bp Marker, TV- ToLCV Vellayani and BV- BYVMV Vellayani isolate and (b) M-100 bp marker and PM- PYVMV Mukhathala isolate

25). All the virus isolates from infected leaf samples of okra, tomato and pumpkin could amplify the product size ~575 bp with AV/AC primer (Plate 26) and an amplicon of size ~520 bp with the DENG primer (Plate 27 and 28) which confirmed the presence of the begomoviruses (Table 19).

### 4.6.3 Characterization of coat protein gene of BYVMV, ToLCV and PYVMV

Partial molecular characterization was undertaken by sequencing coat protein amplicons at Agrigenome Pvt. Ltd. Kochi, and BLAST analysis was undertaken in NCBI website. All the isolates of the begomoviruses amplified with AV/AC were subjected to sequencing followed by BLASTn analysis in NCBI and nucleotide identity with the already submitted accessions of the viruses were assessed.

Nucleotide sequence analysis of coat protein gene of BYVMV isolates from okra shared similarity with *Okra enation leaf curl virus* (99.42 % - 97.91 %) (MK084768.1, KY216075.1 and KT898975.1). The ToLCV isolates of Vellayani and Neyyattinkara shared highest similarity to bipartite Begomovirus *Tomato leaf curl New Delhi virus* (96.89 % - 96.71 %) (KM269360.1, KM269359.1 and KM363407.1); while the rest of the ToLCV isolates were found to be more similar to monopartite begomoviruses *viz., Tomato leaf curl Kerala virus* (99.05 % - 95.82 %) (KY511140.1, MG904838.1, MK087122.1 and AJ810367.1) *Tomato leaf curl Gujarat virus* (98.11 %) (AY234383.1, KY799159.1 and KY799159.1) and *Tomato leaf curl Patna virus* (91.86 %) (JN381198.1and KU933675.1). The PYVMV isolates from pumpkin shared similarity to *Squash leaf curl China virus* (SLCCNV) (98.63 %) (Table 20-22).

The BLASTx analysis for detecting the type of protein amplified, confirmed that the region of the genome amplified were coat protein of the concerned viruses. BYVMV isolates shared maximum similarity to CP of *Okra enation leaf curl virus* isolates (100 % - 97.2 %) (ALN96436.1, ALN96436.1 and ALN96435.1) (Table 23).

Table 19. PCR amplification of Begomovirus isolates from the infected okra, tomato and pumpkin leaf samples using AV/AC and DENG primers

| Crop | Begomovirus isolates | Primers | |
|---|---|---|---|
| | | AV/AC | DENG |
| Okra | BYVMV Vellayani | + | + |
| | BYVMV Neyyattinkara | + | + |
| | BYVMV Nedumangad | + | + |
| | BYVMV Chirayinkeezhu | + | + |
| | BYVMV Mukhathala | + | + |
| | BYVMV Kottarakkara | + | + |
| | BYVMV Punalur | + | + |
| Tomato | ToLCV Vellayani | + | + |
| | ToLCV Neyyattinkara | + | + |
| | ToLCV Nedumangadu | + | + |
| | ToLCV Chirayinkeezhu | + | + |
| | ToLCV Tavanur | + | + |
| | ToLCV Mukhathala | + | + |
| | ToLCV Punalur | + | + |
| Pumpkin | PYVMV Kasaragod | + | + |
| | PYVMV Pappanchani | + | + |
| | PYVMV Tavanur | + | + |
| | PYVMV Mukhathala | + | + |

Table 20. Blast n analysis of the coat protein sequences of BYVMV isolates from different locations of Kerala

| Sl. No. | Begomovirus isolates | Similarity to other begomoviruses | Percentage similarity | Accession uumber |
|---|---|---|---|---|
| 1. | BYVMV Vellayani | *Okra enation leaf curl virus* clone 6.2, complete genome | 99.42% | MK084768.1 |
| | | *Okra enation leaf curl virus* clone 6.1, complete genome | 99.42% | MK084767.1 |
| 2. | BYVMV Neyyattinkara | *Okra enation leaf curl virus* clone 6.2, complete genome | 98.21% | MK084768.1 |
| | | *Okra enation leaf curl virus* clone 6.1, complete genome | 98.21 % | MK084767.1 |
| 3. | BYVMV Nedumangad | *Okra enation leaf curl virus* isolate IB3 clone IB3 AV1 coat protein gene | 98.52% | KY216075.1 |
| | | *Okra enation leaf curl virus* isolate OK86-HR segment DNA A, complete sequence | 97.88% | KT390304.1 |
| 4. | BYVMV Chirayinkeezhu | *Okra enation leaf curl virus* clone 6.2, complete genome | 99.07% | MK084768.1 |
| | | *Okra enation leaf curl virus* clone 6.1, complete genome | 99.07% | MK084767.1 |
| 5. | BYVMV Mukhathala | *Okra enation leaf curl virus* clone 6.2, complete genome | 99.23% | MK084768.1 |
| | | *Okra enation leaf curl virus* clone 6.1, complete genome | 99.23% | MK084767.1 |
| 6. | BYVMV Kottarakkara | *Okra enation leaf curl virus* clone 6.2, complete genome | 99.22% | KT898975.1 |
| | | *Okra enation leaf curl virus* clone 6.1, complete genome | 99.03% | KT390320.1 |
| 7. | BYVMV Punalur | *Okra enation leaf curl virus* clone 6.1, complete genome | 97.91% | MK084767.1 |
| | | *Okra enation leaf curl virus* clone S9C4, complete genome | 97.91% | MK069435.1 |

Table 21. Blast n analysis of the coat protein sequences of ToLCV isolates from different locations of Kerala

| Sl. No. | Begomovirus isolates | Similarity to other begomoviruses | Percentage similarity | Accession number |
|---|---|---|---|---|
| 1. | ToLCV Vellayani | *Tomato leaf curl New Delhi virus* isolate TN POL BG 1 coat protein (AV1) gene, complete cds | 96.71% | KM269360.1 |
| | | *Tomato leaf curl New Delhi virus* isolate TN POL BG4 | 96.52% | KM363407.1 |
| 2. | ToLCV Neyyattinkara | *Tomato leaf curl New Delhi virus* isolate TN POL BG 1 coat protein (AV1) gene, complete cds | 96.89% | KM269360.1 |
| | | *Tomato leaf curl New Delhi virus* isolate TN THON BG 1 coat protein (AV1) gene, complete cds | 96.72% | KM269359.1 |
| 3. | ToLCV Nedumangad | *Tomato leaf curl Kerala virus* isolate Bpl coat protein (AV1) gene, complete cds | 99.05% | KY511140.1 |
| | | *Tomato leaf curl Kerala virus* isolate TC343 segment DNA-A | 98.05% | KF551575.1 |
| 4. | ToLCV Chirayinkee-zhu | *Tomato leaf curl Kerala virus* isolate TC343 segment DNA-A | 97.52% | MG904838.1 |
| | | *Tomato leaf curl Kerala virus* isolate RM43 | 97.11% | JQ693140.1 |
| 5. | ToLCV Tavanur | *Tomato leaf curl Gujarat virus* - [Nepal] segment DNA-A | 98.11% | AY234383.1 |
| | | *Tomato leaf curl Gujarat virus* clone 251ISD segment DNA-A | 97.92% | KY799159.1 |
| 6. | ToLCV Mukhathala | *Tomato leaf curl virus* AV1 gene for coat protein, isolate 28 | 95.82% | MK087122.1 |
| | | *Tomato leaf curl virus strain* Kerala coat protein gene | 95.63% | AJ810367.1 |
| 7. | ToLCV Kottarakkara | *Tomato leaf curl Patna virus* isolate JM-AS-1 coat protein (AV1) gene, complete cds | 91.86% | JN381198.1 |
| | | *Tomato leaf Curl Patna virus*-[Bangladesh:Jamalpur:2014] isolate ToLCV -JB segment DNA-A | 90.71% | KU933675.1 |
| 8. | ToLCV Punalur | *Tomato leaf curl Gujarat virus* - [Nepal] segment DNA-A, complete sequence | 98.11% | AY234383.1 |
| | | *Tomato leaf curl Gujarat virus* clone 251ISD | 97.92% | KY799159.1 |

Table 22. Blast n analysis of the coat protein sequences of PYVMV isolates from different locations of Kerala

| Sl. No. | Begomovirus isolates | Similarity to other begomoviruses | Percentage similarity | Accession number |
|---|---|---|---|---|
| 1. | PYVMV Mukhathala | *Squash leaf curl China virus* isolate TN NGK Pum 1 coat protein (AV1) gene, complete cds | 97.88% | KM260368.1 |
| | | *Squash leaf curl China virus* isolate KP1 segment DNA-A, complete sequence | 97.25% | KF188433.1 |
| 2. | PYVMV Pappanchani | *Coccinia mosaic Virudhunagar virus* segment DNA-A, complete sequence | 93.36% | KY860899.1 |
| | | *Bitter gourd yellow mosaic virus* segment DNA A, complete sequence | 90.43% | MH481856.1 |
| | | Whitefly-transmitted Indian begomovirus from *Parthenium hysterophorus* coat protein (AV1) gene, complete cds | 80.54% | FJ931537.1 |
| | | *Squash leaf curl China virus* isolate TN NGK PUM2 coat protein (AV1) gene, complete cds | 80.54% | AM296494.1 |
| | | *Cotton leaf curl Bangalore virus* DNA, segment DNA-A, complete sequence, isolate: Jabalpur_D | 80.52% | KT390461.1 |
| | | *Tomato leaf curl New Delhi virus* clone pCalH2 segment DNA-A, complete sequence | 80.51% | KC202818.1 |
| 3. | PYVMV Kasaragod | *Squash leaf curl China virus* isolate TN NGK Pum 1 coat protein (AV1) gene, complete cds | 98.63% | KM260368.1 |
| | | *Squash leaf curl China virus* isolate TN NGK CUCU 1 coat protein (AV1) gene, complete cds | 98.24% | KM260367.1 |
| 4. | PYVMV Tavanur | *Squash leaf curl China virus* isolate TN UDU PUM 2 coat protein (AV1) gene, complete cds | 98.33% | KM269348.1 |
| | | *Squash leaf curl China virus* isolate TN UDU PUM 1 coat protein (AV1) gene, complete cds | 98.33% | KM269347.1 |

Table 23. Blast x analysis of coat protein sequences of BYVMV isolates from different locations of Kerala

| Sl. No. | Begomovirus isolates | Similarity to begomovirus protein | Percentage similarity | Accession number |
|---|---|---|---|---|
| 1. | BYVMV Vellayani | coat protein [*Okra enation leaf curl virus]* | 98.28% | ALN96434.1 |
| | | coat protein [*Okra enation leaf curl virus]* | 98.28% | ALN96435.1 |
| 2. | BYVMV Neyyattinkara | coat protein [*Okra enation leaf curl virus]* | 99.39% | ALN96436.1 |
| | | coat protein [*Okra enation leaf curl virus]* | 99.39% | ALN96435.1 |
| 3. | BYVMV Nedumangad | coat protein [*Okra enation leaf curl virus]* | 99.39% | ALN96436.1 |
| | | coat protein [*Okra enation leaf curl virus]* | 99.39% | ALN96435.1 |
| 4. | BYVMV Chirayinkeezhu | coat protein [*Okra enation leaf curl virus]* | 95.72% | ALN96435.1 |
| | | coat protein [*Okra enation leaf curl virus]* | 95.72% | ALB26312.1 |
| 5. | BYVMV Mukhathala | coat protein [*Okra enation leaf curl virus]* | 100% | ALN96436.1 |
| | | coat protein [*Okra enation leaf curl virus]* | 100% | ALN96435.1 |
| 6. | BYVMV Kottarakkara | coat protein [*Okra enation leaf curl virus]* | 98.85% | ALN96434.1 |
| | | coat protein [*Okra enation leaf curl virus]* | 98.85% | ALN96435.1 |
| 7. | BYVMV Punalur | coat protein [*Okra enation leaf curl virus]* | 96.88% | ALN96434.1 |
| | | coat protein [*Okra enation leaf curl virus* | 96.88% | ALN96435.1 |

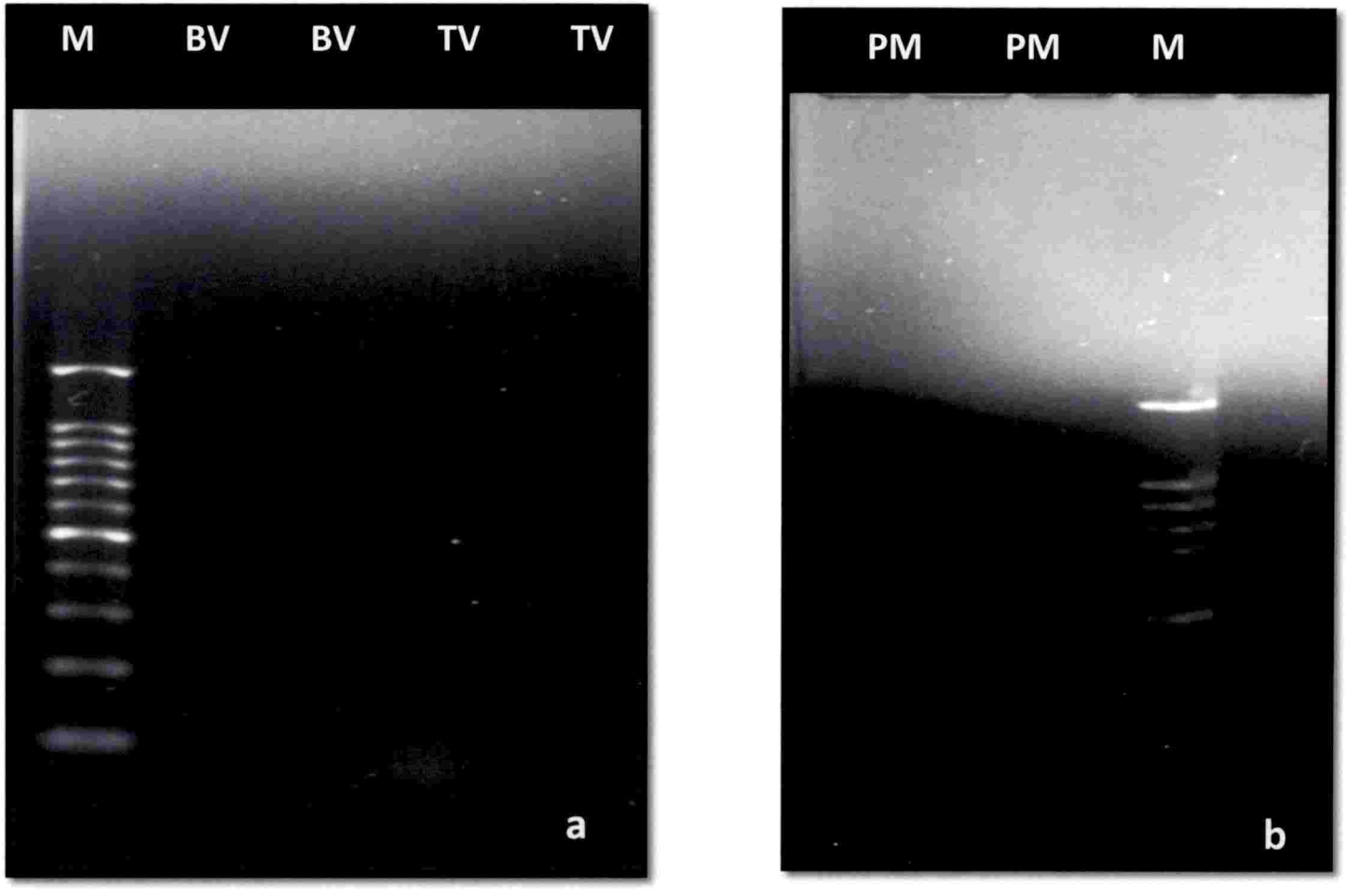


Plate 25. Amplification of coat protein gene of Begomoviruses isolated from the mature seeds of the infected okra, tomato and pumpkin (dried mature seeds) using AV/AC and DENG primer (a) M-100 bp marker, BM- BYVMV Vellayani and TV- T oLCVVellayani isolate; and (b) M-100 bp marker and PM- PYVMV Mukhathala isolate.

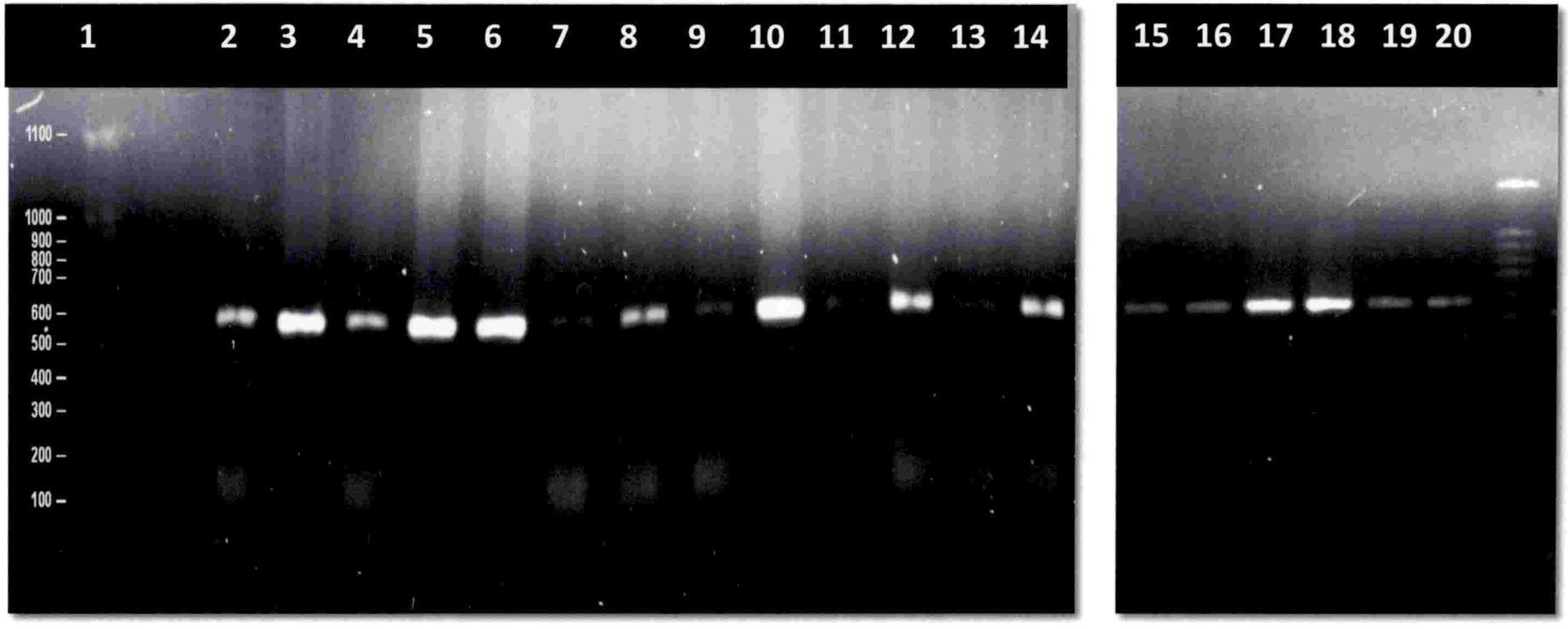


Plate 26. Amplification of coat protein gene (575 bp) of Begomoviruses isolated from the infected leaf samples of okra and tomato using AV/AC primer. Lane 1-100 bp Marker, 2-BYVMV Vellayani, 3-BYVMV Neyyattinkara, 4-BYVMV Nedumangad, 5-BYVMV Chirayinkeezhu, 6-BYVMV Mukhathala, 7-BYVMV Punalur, 8-BYVMV Kottarakkara, 9-ToLCV Tavanur, 10-TolCV Vellayani, 11-ToLCVChirayinkeezhu, 12-ToLCV Neyyattinkara, 13-ToLCV Nedumangad, 14-ToLCV Mukhathala, 15-ToLCV Kottarakkara, 16-ToLCV Punalur, 17-PYVMV Kasaragod, 18-PYVMV Tavanur, 19-PYVMV Mukhathala and 20-PYVMV Pappanchani.

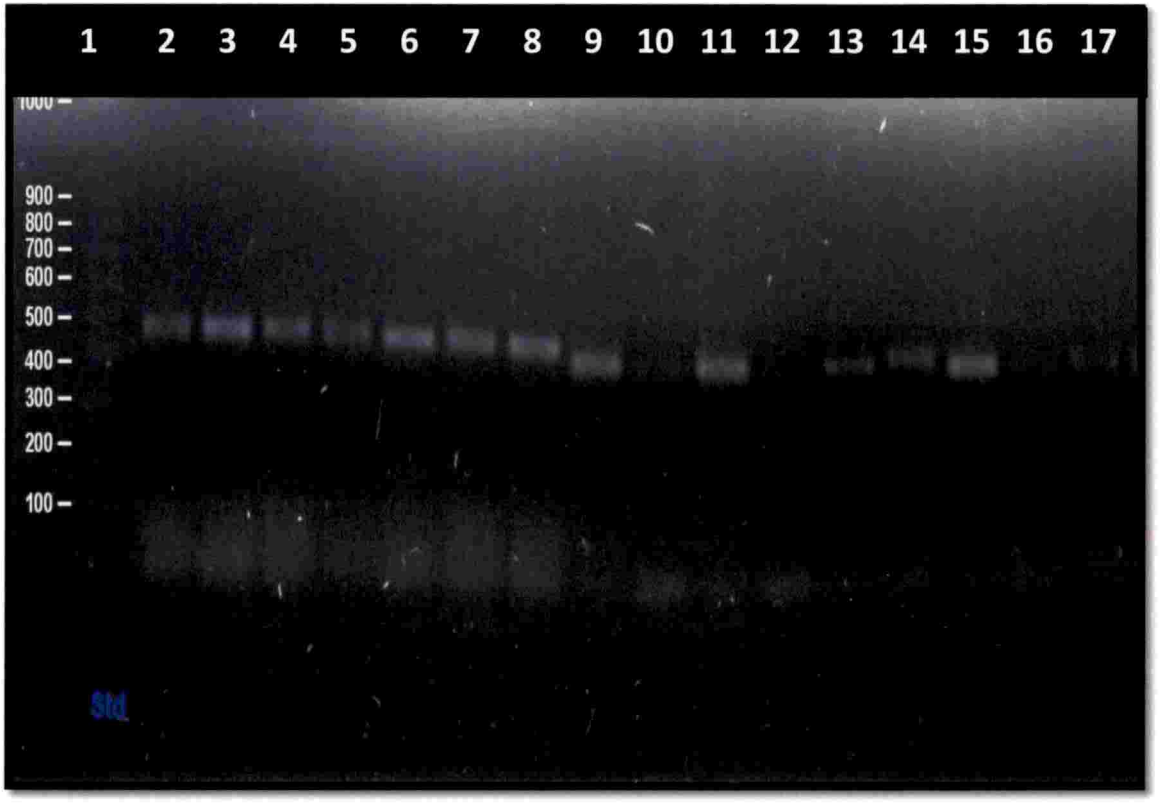


Plate 27 . Amplification of coat protein gene (520 bp) of Begomoviruses isolated from the infected okra, tomato and pumpkin leaf samples using DENG primer. Lane 1-100 bp Marker, 2-BYVMV Vellayani, 3-BYVMV Neyyattinkara, 4-BYVMV Nedumangad, 5-BYVMV Chirayinkeezhu, 6-BYVMV Mukhathala, 7-BYVMV Punalur, 8-BYVMV Kottarakkara, 9-ToLCV Tavanur, 10-ToLCV Vellayani, 11-ToLCV Chirayinkeezhu, 12-ToLCV Neyyattinkara, 13-ToLCV Nedumangad, 14-ToLCVMukhathala, 15-ToLCV Kottarakkara, 16-TolCV Punalur, 17-PYVMV Pappanchani

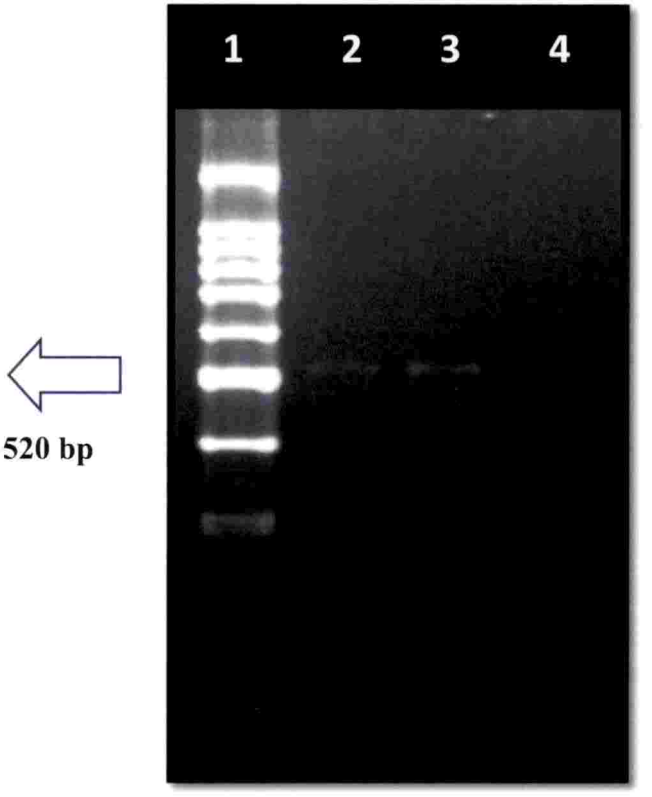


Plate 28. Amplification of coat protein gene (520 bp) of Begomoviruses isolated from the infected okra, tomato and pumpkin leaf samples using DENG primer. Lane: 1-100 bp Marker, 2-PYVMV Mukhathala, 3-PYVMV Kasaragod and 4-PYVMV Tavanur

The ToLCV isolates shared similarity to CP of *Squash leaf curl China virus* isolates (99.43 % - 98.85 % with AIU98070.1, AIW42880.1 and AIU98071.1), *Tomato leaf curl Kerala virus* (98.64 % - 98.26 %) (AHA82161.1, ARK38518.1 and AWM31259.1) and *Tomato leaf curl Gujarat virus* (90.70 %; AAL78666.1) (Table 24). While PYVMV isolates shared highest similarity to CP of *Tomato leaf curl New Delhi virus* (97.69 % - 95.60 %; AJY58643.1, AMX27950.1 and CBJ17668.1) and *Squash leaf curl China virus* (97.69 %; AIU98071.1) (Table 25).

### 4.6.4 Full genome amplification of DNA-A of BYVMV, ToLCV and PYVMV

#### 4.6.4.1 Rolling circle amplification (RCA)

The full genome (2.7 kb) amplification of the three begomoviruses were done by rolling circle amplification (RCA) method using random hexaprimers and φ DNA polymerase. RCA yielded ~2.7 kb sized full genome in case of all the three viruses *viz.*, BYVMV, ToLCV and PYVMV (Plate 29).

#### 4.6.4.2 Restriction with different endonucleases

The RCA products of the representative samples (BYVMV Vellayani, ToLCV Vellayani and PYVMV Mukhathala) of three begomoviruses were put under restriction fragment length analysis (RFLA) using five restriction enzymes *viz.*, *Bam*HI, *Hind*III, *Kpn*I, *Sal*I *and Xba*I (Table 26). RFL analysis of the RCA products of the three viruses revealed that 2.7 kb sized intact full genome could be obtained on restriction by *Hind*III enzyme in case of BYVMV. While in case of ToLCV *Hind*III, *Kpn*I and *Sal*I yielded intact full genome and both *Bam*HI and *Xba*I yeilded full genome of PYVMV (Plate 29).

#### 4.6.4.3 Full genome amplification using primers

In order to characterize the full genome of ~2.7 kb size, the three begomovirus *viz.*, BYVMV, ToLCV and PYVMV full genome was first amplified using three sets

Table 24. Blast x analysis of coat protein sequences of ToLCV isolates from different locations of Kerala

| Sl. No. | Begomovirus isolates | Similarity to begomovirus protein | Percentage similarity | Accession number |
|---|---|---|---|---|
| 1. | ToLCV Vellayani | coat protein [*Squash leaf curl China virus*] | 98.85% | AIU98070.1 |
| | | coat protein [ *Squash leaf curl China virus*] | 98.85% | AIU98071.1 |
| 2. | ToLCV Neyyattin-kara | coat protein [ *Squash leaf curl China virus*] | 99.43% | AIU98070.1 |
| | | coat protein [ *Squash leaf curl China virus*] | 98.86% | AIW42880.1 |
| 3. | ToLCV Nedumangad | coat protein [*Tomato leaf curl Kerala virus*] | 98.26% | AWM31259.1 |
| | | AV1 [*Tomato leaf curl Kerala virus*] | 96.07% | AHA82161.1 |
| 4. | ToLCV Chirayin-keezhu | coat protein [*Tomato leaf curl Kerala virus*] | 98.64% | ARK38518.1 |
| | | AV1 [*Tomato leaf curl Kerala virus*] | 98.64% | AHA82161.1 |
| 5. | ToLCV Tavanur | coat protein [*Tomato leaf curl virus*] | 90.70% | CAH17800.1 |
| | | coat protein [*Tomato leaf curl Gujarat virus*-[Vadodara]] | 90.70% | AAL78666.1 |
| 6. | ToLCV Mukhathala | coat protein [*Ageratum yellow vein Sri Lanka virus*] | 97.08% | NP_148952.1 |
| | | coat protein [*French bean severe leaf curl virus*] | 97.08% | QCE30481.1 |
| 7. | ToLCV Kottarakkara | coat protein [*Tobacco leaf curl Pusa virus*] | 97.60% | AJE24731.1 |
| | | coat protein [*Tobacco leaf curl Pusa virus*] | 97.60% | YP_003934911.1 |
| 8. | ToLCV Punalur | coat protein [*Tomato leaf curl virus*] | 90.70% | CAH17800.1 |
| | | coat protein [*Tomato leaf curl Gujarat virus*-[Vadodara]] | 90.70% | AAL78666.1 |

Table 25. Blast x analysis of coat protein sequences of PYVMV isolated from different locations of Kerala

| Sl. No. | Begomovirus isolates | Similarity to begomovirus protein | Percentage similarity | Accession number |
|---|---|---|---|---|
| 1. | Pumpkin Mukhathala | coat protein [*Tomato leaf curl New Delhi virus]* | 95.60% | CBJ17668.1 |
| | | coat protein [*Tomato leaf curl New Delhi virus]* | 95.60% | AMX27950.1 |
| 2. | Pumpkin Pappanchani | AV1 [*Coccinia mosaic Virudhunagar virus*] | 90.55% | ATO88007.1 |
| | | *Tomato leaf curl New Delhi virus* clone pCalH2 segment DNA-A, complete sequence | 80.51% | KC202818.1 |
| 3. | Pumpkin Kasaragod | coat protein [*Squash leaf curl China virus*] | 97.69% | AIU98071.1 |
| | | coat protein [*Tomato leaf curl New Delhi virus]* | 97.69% | AJY58643.1 |
| 4. | Pumpkin Tavanur | coat protein [Begomovirus pumpkin/SuphanBuri/Mar2008] | 96.22% | ACN62171.1 |
| | | coat protein [*Tomato leaf curl New Delhi virus]* | 96.22% | AMB18871.1 |

Table 26. Restriction enzymes tested in the study with their specific restriction sites

| Restriction enzyme used | Restriction site (5'-3') |
|---|---|
| BamHI | G↓GATCC |
| HindIII | A↓AGCTT |
| SalI | G↓TCGAC |
| KpnI | GGTAC↓C |
| XbaI | T↓CTAGA |

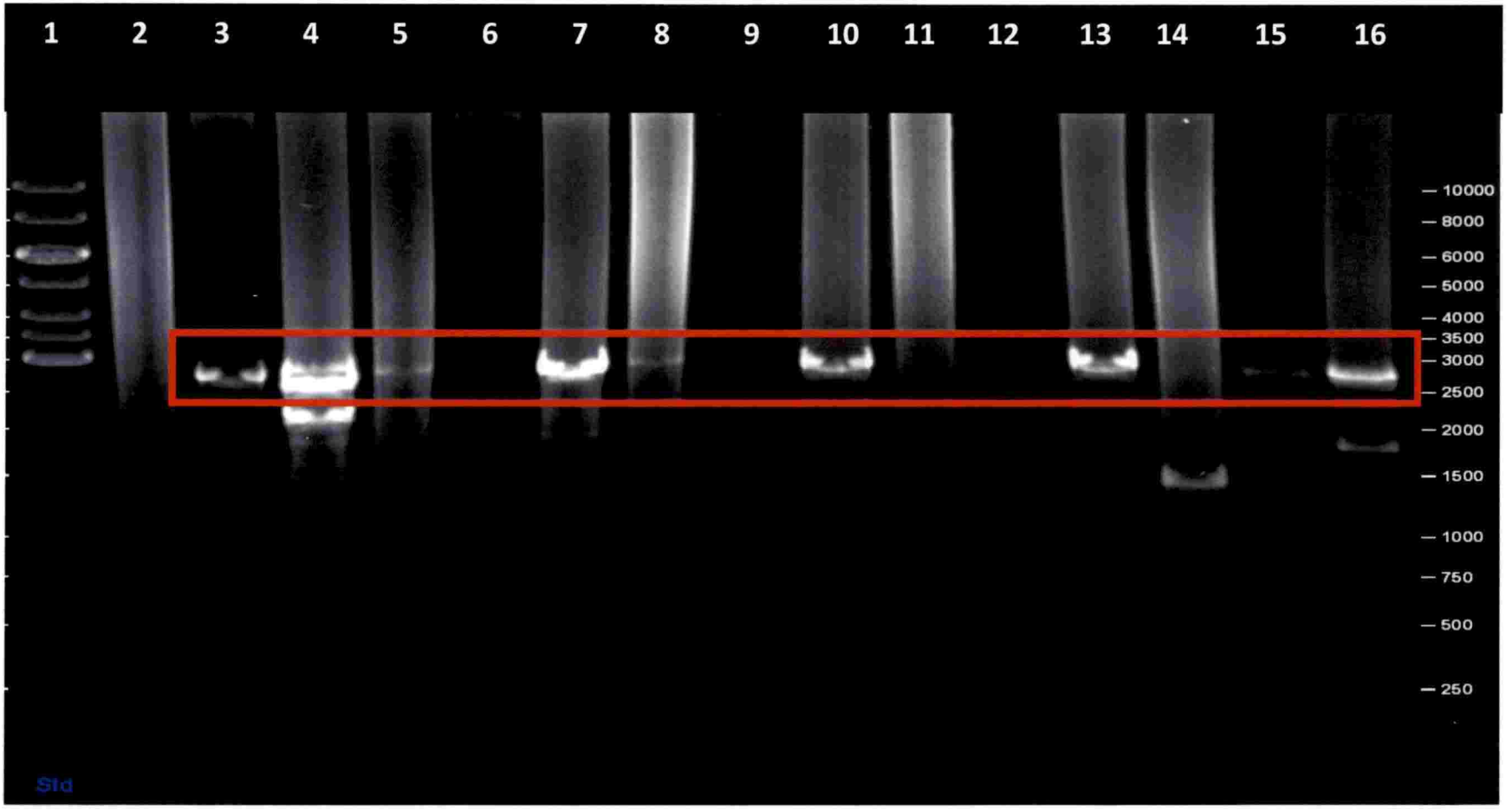


Plate 29. Amplification of full-length genome (~2.7 kb size) of Begomovirus isolates of okra, tomato and pumpkin after restriction with endonucleases *viz., Bam*HI, *Hind*III, *Sal*I, *Kpn*I and *Xba*I. Lane 1: Molecular marker, 2: BYVMV *Bam*HI, 3: PYVMV *Bam*HI, 4: ToLCV *Bam*HI, 5: BYVMV *Hind*III, 6: PYVMV *Hind*III, 7: ToLCV *Hind*III, 8: BYVMV *Kpn*I, 9: PYVMV *Kpn*I, 10: ToLCV *Kpn*I, 11: BYVMV *Sal*I, 12: PYVMV *Sal*I, 13: ToLCV *Sal*I, 14: BYVMV *Xba*I, 15: PYVMV *Xba*I and 16: ToLCV *Xba*I.

of degenerative overlapping primers developed using multiple sequence alignment (MSA) method (Clustal W software). The primers were developed by MSA of a total of 22 selected begomovirus sequences of BYVMV, ToLCV and SLCCNV obtained from NCBI database that expressed maximum similarity to CP gene sequence of the corresponding viruses (Fig. 1) (Table 27). The three sets of primers designed with their sequences and product size of the amplicons they produce were as follows:

BIN 1 (F): 3' TATGKCGAAGCGWSCHSC 5'; ~ 1000 bp product size

BIN 2 (R): 3' TGGGRVATDCACMARTGTKT 5';

BIN 3 (F): 3' TATATGGCHTGTACNCAYG 5'; ~1300 bp product size

BIN 4 (R): 3' CRAACATHCAGGGAGCTAA 5';

BIN 5 (F): 3' GATCKDCCRTCGAYYTGRA 5'; ~900 bp product size

BIN 6 (R): 3' TTTGTGACGCGGGCAATGG 5';

[R=A+G; Y=C+T; M=A+C; K=G+T; S=G+C; W=A+T; H=A+T+C; D=G+A+T; N=A+C+G+T and V=G+A+C]

The three sets of primers [BIN(F)-1, BIN(R)-2, BIN(F)-3, BIN(R)-4, BIN(F)-5 and BIN(R)-6] did amplify amplicons of size 1300 bp, 1000 bp and 900 bp in BYVMV, ToLCV and PYVMV (Plate 30). The amplicons were eluted from the gel using gel extraction kit (GeneJET Gel Extraction Kit, Cat: #K0691, #K0692; Thermo Scientific) and sequenced.

### 4.6.5 Characterization of full genome of DNA-A of BYVMV, ToLCV and PYVMV

The full genome sequences of the three virus isolates *viz.,* BYVMV Vellayani, ToLCV Vellayani and PYVMV Mukhathala obtained were BLAST analyzed in NCBI site. The sequence analysis conveyed the fact that the BYVMV isolate of

Table 27. List of accessions of full genome sequences used for the multiple sequence alignment for designing primers for amplification of DNA-A genome of Begomoviruses infecting okra, tomato and pumpkin (Continued)

| Sl. No. | Accession number | Virus | Host | Country / Place |
|---|---|---|---|---|
| 1. | DQ026296.2 | *Squash leaf curl China virus* | Pumpkin | Lucknow |
| 2. | AY184487.3 | *Squash leaf curl China virus* | Pumpkin | Coimbatore, Tamil Nadu |
| 3. | KF188433.1 | *Squash leaf curl China virus* | *Benincasa hispida* | India |
| 4. | KP195265.1 | *Tomato leaf curl New Delhi virus* | Tomato | Kalakadu, Andhra Pradesh |
| 5. | KY979591.1 | *Tomato leaf curl New Delhi virus* | Tomato | Tamil Nadu |
| 6. | KT426907.1 | *Tomato leaf curl New Delhi virus* | Ridge gourd | Trivandrum, Kerala |
| 7. | KP868764.1 | *Tomato leaf curl New Delhi virus* | Bitter gourd | Phalaghat, Kerala |
| 8. | NC_003418.1 | *Bhendi yellow vein mosaic virus* | Okra | Madurai |
| 9. | GU112060.1 | *Bhendi yellow vein mosaic virus* | Okra | India: Kerala |
| 10. | GU112080.1 | *Bhendi yellow vein mosaic virus* | Okra | India: Coimbatore, Tamil Nadu |
| 11. | GU112062.1 | *Bhendi yellow vein mosaic virus* | Okra | India: Palaghat, Kerala |
| 12. | GU112072.1 | *Bhendi yellow vein mosaic virus* | Okra | India: Palaghat, Kerala |

Table 27. List of accessions of full genome sequences used for the multiple sequence alignment for designing primers for amplification of DNA-A genome of Begomoviruses infecting okra, tomato and pumpkin (Continued)

| Sl. No. | Accession number | Virus | Host | Country / Place |
|---|---|---|---|---|
| 13. | JQ326268.1 | *Bhendi yellow vein mosaic virus* | Okra | India : Pandarahalli, Tamil Nadu |
| 14. | KJ462083.1 | *Mesta yellow vein mosaic virus* | Okra | India :Varanasi, Uttar Pradesh |
| 15. | MK084768.1 | *Okra enation leaf curl virus* | Okra | India : Maharashtra |
| 16. | KX698092.1 | *Okra enation leaf curl virus* | Okra | Sri Lanka |
| 17. | KM383734.1 | *Tobacco leaf curl Pusa virus* | Tomato | Bangladesh: Rajshah |
| 18. | KU933675.1 | *Tomato leaf Curl Patna virus* | Tomato | Bangladesh: Jamalpur |
| 19. | AF314144.1 | *Ageratum yellow vein Sri Lanka virus* | Ageratum | Sri Lanka |
| 20. | AY234383.1 | *Tomato leaf curl Gujarat virus* | Tomato | India |
| 21. | KY926898.1 | *Tomato leaf curl Kerala virus* | *Populus alba* | Pakistan |
| 22. | LT556075.1 | *Tomato leaf curl Kerala virus* | Tomato | Pakistan |

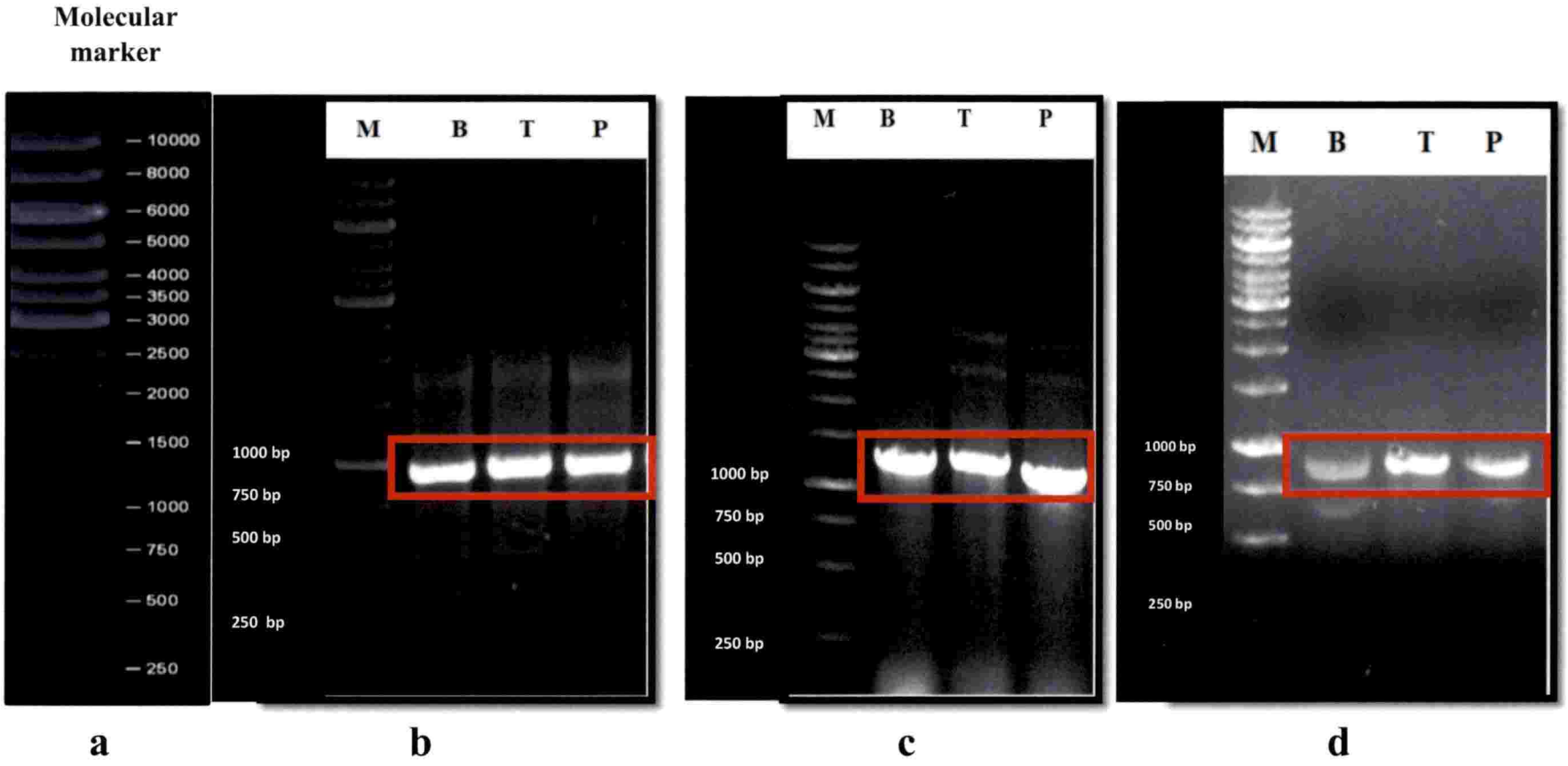


Plate 30. Agarose gel of PCR amplifications of DNA-A genome of BYVMV, ToLCV and PYVMV using three sets of overlapping degenerative primers. (a) Molecular marker; (b) M: marker, B: BYVMV, T: ToLCV and P: PYVMV amplified using primers BIN 1 and BIN 2 with product size 1000 bp; (c) M: marker, B: BYVMV, T: ToLCV and P: PYVMV amplified using primer BIN 3 and BIN 4 with product size 1300 bp and (d) M: marker, B: BYVMV, T: ToLCV and P: PYVMV amplified using primer BIN 5 and BIN 6 with product size 900 bp.

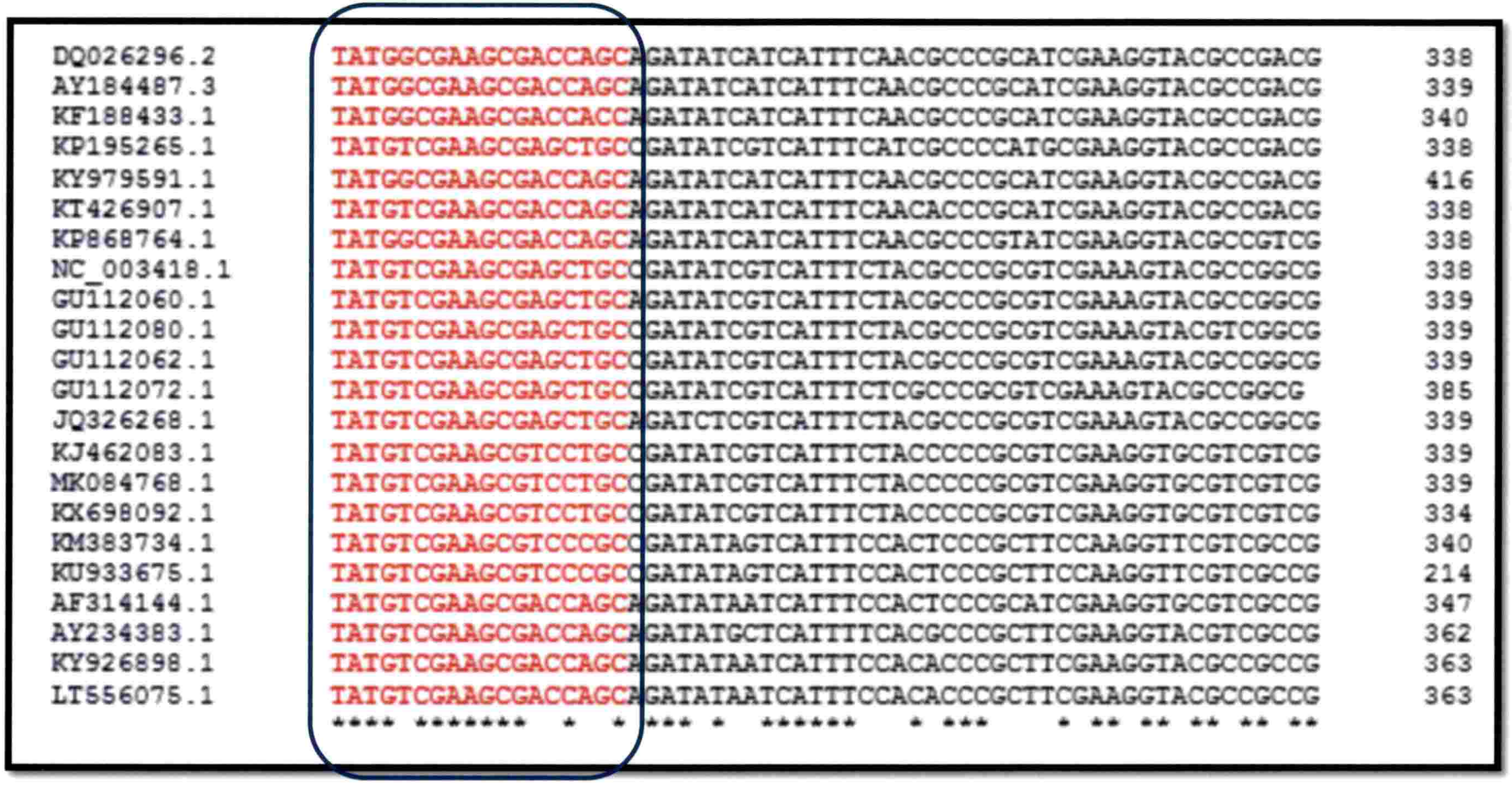


Fig 1. Multiple alignments of selected 22 Begomovirus sequences in NCBI database for designing primers to amplify DNA-A full genome of BYVMV, ToLCV and PYVMV.

Vellayani was more similar to *Bhendi yellow vein mosaic virus* isolate OY173 segment DNA-A, complete sequence (Acc. No. - JQ326268.1) with 95.04 per cent similarity confirming that it is BYVMV involved in BYVMD in Kerala and named the virus as BYVMV Vellayani. Similarly, BLAST analysis of ToLCV isolate of Vellayani shared highest similarity to *Tomato leaf curl New Delhi virus* isolate TC237 segment DNA-A, complete sequence (Acc. No.- KF551582.1) with 92.89 per cent similarity, hence named as ToLCNDV Vellayani. The PYVMV isolate of Mukhathala shared maximum similarity with *Squash leaf curl China virus* - [Pumpkin :Coimbatore] segment DNA-A, complete sequence (Acc. No.- AY184487.3) with 98.09 per cent similarity and hence named as SLCCNV Mukhathala (Table 28).

Full genome of DNA-A of BYVMV, ToLCNDV and SLCCNV were translated to protein sequences and analyzed in open reading frame (ORF) finder (**https://web.expasy.org/translate/**) and the different ORFs present in the genome were recorded. The DNA-A of BYVMV Vellayani and ToLCNDV Vellayani isolates consisted of seven ORF arranged in such that ORFs AV1 and AV2 were present in the sense strand while the other five ORFs (AC1, AC2, AC3, AC4 and AC5) were found to be present in the complement strand. In case of Mukhathala of SLCCNV's DNA-A genome consisted of only 6 ORF's of which two of them (AV1 and AV2) were present in sense strand and the rest (AC1 to AC4) were present in the complement strand. The different ORF coded for their respective proteins as mentioned in the Table 29-31.

## 4.7 GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS

### 4.7.1 Genetic diversity and phylogenetic analysis of coat protein gene

Phylogenetic relationship among coat protein sequences of 19 begomoviruses isolates were constructed using the neighbor-joining method in the MEGA 7.0.26

Table 28. List of Begomoviruses showing highest similarity to DNA-A sequence of the Begomovirus isolates of okra, tomato and pumpkin

| Sl. No. | Begomovirus isolate | Virus name | Accession number | Percentage similarity |
|---|---|---|---|---|
| 1. | BYVMV-Vellayani | *Bhendi yellow vein mosaic virus* isolate OY173 segment DNA-A, complete sequence | JQ326268.1 | 95.04 |
| 2. | ToLCV-Vellayani | *Tomato leaf curl New Delhi virus* isolate TC237 segment DNA-A, complete sequence | KF551582.1 | 92.89 |
| 3. | PYVMV-Mukhathala | *Squash leaf curl China virus* - [Pumpkin: Coimbatore] segment DNA-A, complete sequence | AY184487.3 | 98.09 |

Table 29. Genome organization of DNA-A of BYVMV Vellayani isolate

| Virus | Genome size | Open Reading Frame (ORF) | Length of ORF | Coded protein | Coding strand |
|---|---|---|---|---|---|
| BYVMV | 2735 bases | ORF 1 (AV2) | 122 AA | Pre coat protein | Sense strand |
| | | ORF 2 (AV1) | 256 AA | Coat protein | Sense strand |
| | | ORF 3 (AC5) | 118 AA | - | Complement |
| | | ORF 4 (AC3) | 134 AA | Replication enhancer protein | Complement |
| | | ORF 5 (AC2) | 150 AA | Transcriptional activator protein | Complement |
| | | ORF 6 (AC1) | 363 AA | Replicase protein | Complement |
| | | ORF 7 (AC4) | 100 AA | - | Complement |

Table 30. Genome organization of DNA-A of ToLCNDV Vellayani isolate

| Virus | Genome size | Open Reading Frame | Length | Coded protein | Coding strand |
|---|---|---|---|---|---|
| ToLCNDV | 2740 bases | ORF 1 (AV2) | 115 AA | Pre coat protein | Sense strand |
| | | ORF 2 (AV1) | 256 AA | Coat protein | Sense strand |
| | | ORF 3 (AC5) | 161 AA | - | Complement |
| | | ORF 4 (AC3) | 136 AA | Replication enhancer protein | Complement |
| | | ORF 5 (AC2) | 139 AA | Transcriptional activator protein | Complement |
| | | ORF 6 (AC1) | 361 AA | Replicase protein | Complement |
| | | ORF 7 (AC4) | 58 AA | - | Complement |

Table 31. Genome organization of DNA-A of SLCCNV Mukhathala isolate

| Virus | Genome size | Open Reading Frame | Length | Coded protein | Coding strand |
|---|---|---|---|---|---|
| SLCCNV | 2738 bases | ORF 1 (AV2) | 112 AA | Pre coat protein | Sense strand |
| | | ORF 2 (AV1) | 256 AA | Coat protein | Sense strand |
| | | ORF 3 (AC3) | 136 AA | Replication enhancer protein | Complement |
| | | ORF 4 (AC2) | 134 AA | Transcriptional activator protein | Complement |
| | | ORF 5 (AC1) | 361 AA | Replicase protein | Complement |
| | | ORF 6 (AC4) | 58 AA | - | Complement |

software program. The analysis revealed that all the three viruses *viz.*, BYVMV, ToLCV and PYVMV isolates were clustered together with similar virus isolates confirming that they were more related to each other compared to the other viruses.

In case of ToLCV, Vellayani and Neyyattinkara isolates were found to be clustered together with PYVMV isolates while the rest of the ToLCV isolates were clustered together separately confirming that they were more related to each other compared to the above mentioned two isolates of ToLCV. Further, a few ToLCV isolates exhibited similarity to BYVMV isolates, suggesting ToLCV could be a connecting link between BYVMV and PYVMV (Fig. 2). Parsimony analysis tree depicting the history of evolution among the begomoviruses also conveyed the same observations as that of the phylogenetic analysis (Fig. 3).

Genetic distance analysis also confirmed that similar virus isolates were more related since their genetic distance value were found to be smaller. Among the 19 begomovirus isolates the highest genetic distance value was found to be between Punalur isolate of BYVMV and Pappanchani isolate of PYVMV, while the least value i.e., cent similarity was found between ToLCV isolates from Punalur and Tavanur (Fig. 4).

### 4.7.2 Genetic diversity and phylogenetic analysis of full genome of the three begomoviruses

Phylogenetic relationship of each of the virus isolates were compared with the similar virus isolates of the virus from within and outside the country present in the NCBI database. The sequences were selected randomly for the phylogenetic analysis. The phylogenetic tree constructed using the neighbor-joining method in the MEGA 6 software program revealed that in case of Vellayani isolate of BYVMV, the DNA-A genome sequenced was found to be more related to other BYVMV isolates from Kerala, Tamil Nadu and Karnataka; the states that are of the same geographical

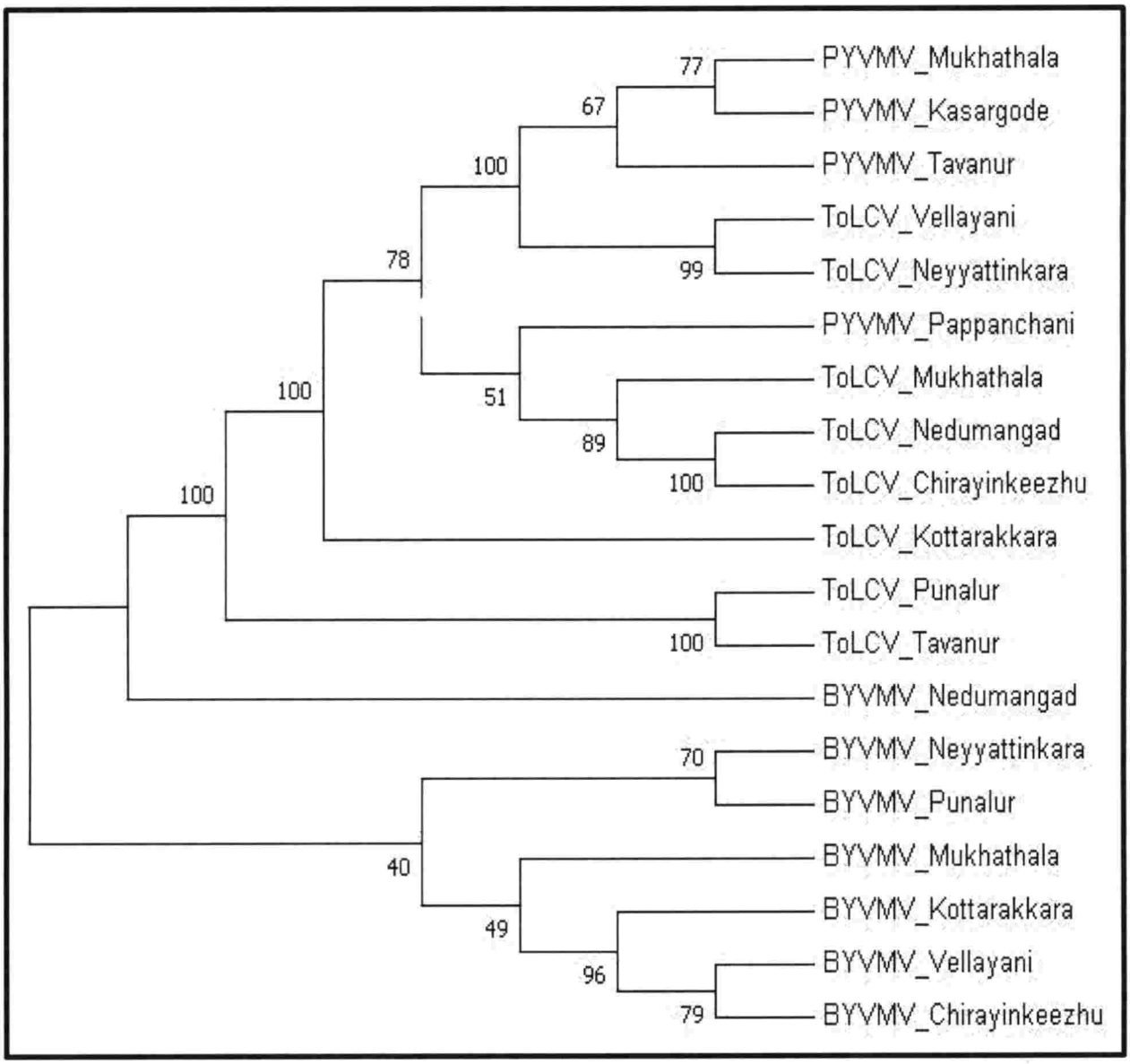


Fig. 2. Phylogeny tree constructed based on nucleotide sequences of coat protein gene of Begomovirus isolates from Kerala using MEGA 7.0.26 software program. The bootstrap scores in percent were denoted at nodes (500 replicates).

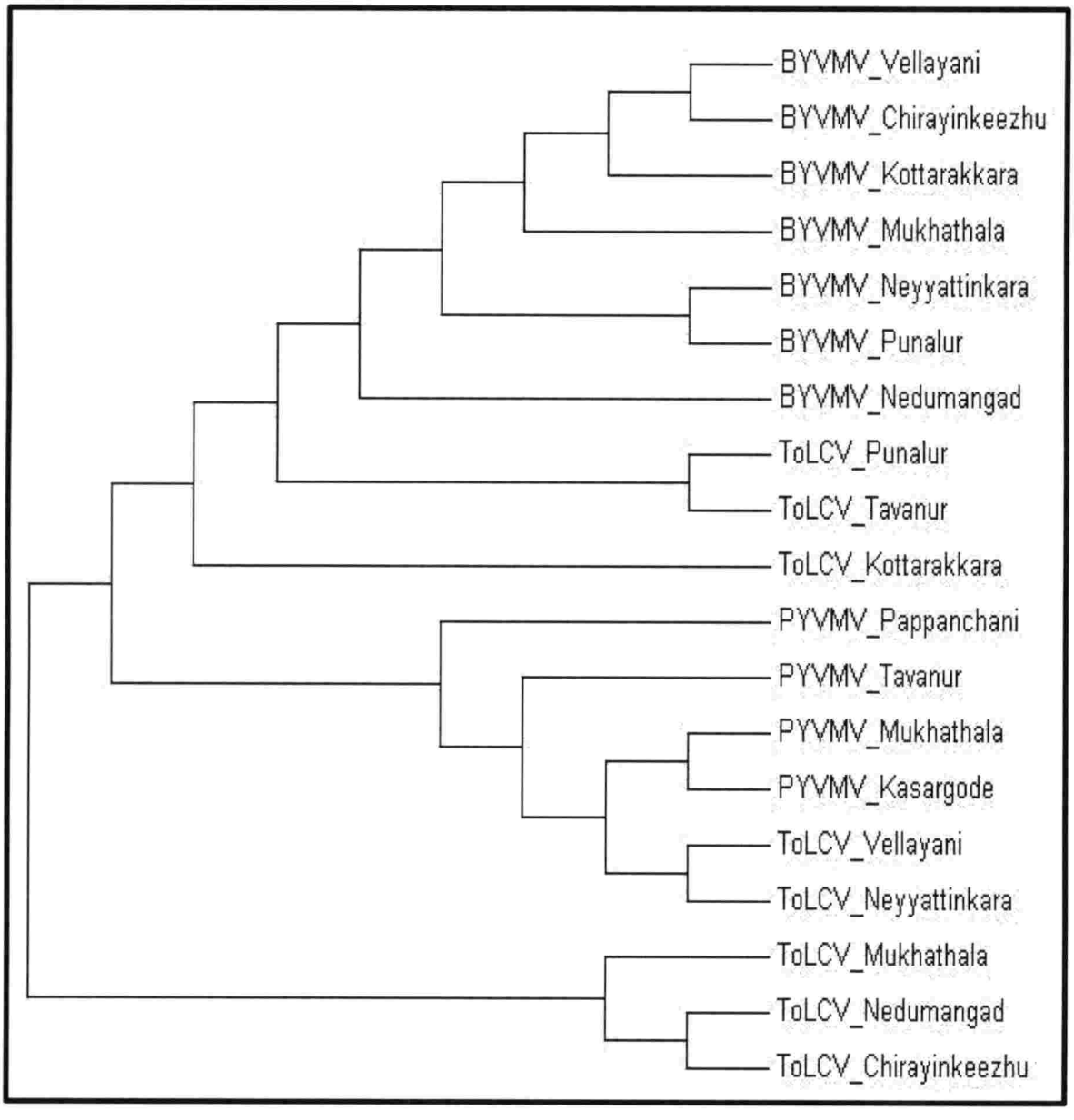


Fig. 3. Parsimony analysis of coat protein sequence of different isolates of BYVMV, ToLCV and PYVMV (using AV/AC primer) from Kerala in MEGA 7.0.26 software program

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. BYVMV_Vellayani | | | | | | | | | | | | | | | | | | | |
| 2. BYVMV_Neyyattinkara | 0.052 | | | | | | | | | | | | | | | | | | |
| 3. BYVMV_Nedumangad | 0.043 | 0.040 | | | | | | | | | | | | | | | | | |
| 4. BYVMV_Chirayinkeezhu | 0.014 | 0.052 | 0.037 | | | | | | | | | | | | | | | | |
| 5. BYVMV_Mukhathala | 0.029 | 0.029 | 0.031 | 0.029 | | | | | | | | | | | | | | | |
| 6. BYVMV_Kottarakkara | 0.017 | 0.046 | 0.037 | 0.023 | 0.020 | | | | | | | | | | | | | | |
| 7. BYVMV_Punalur | 0.068 | 0.052 | 0.055 | 0.071 | 0.049 | 0.059 | | | | | | | | | | | | | |
| 8. PYVMV_Mukhathala | 0.484 | 0.452 | 0.456 | 0.477 | 0.472 | 0.479 | 0.500 | | | | | | | | | | | | |
| 9. PYVMV_Tavanur | 0.435 | 0.437 | 0.418 | 0.421 | 0.431 | 0.426 | 0.488 | 0.086 | | | | | | | | | | | |
| 10. PYVMV_Kasargode | 0.417 | 0.423 | 0.398 | 0.406 | 0.412 | 0.417 | 0.480 | 0.064 | 0.067 | | | | | | | | | | |
| 11. PYVMV_Pappanchani | 0.471 | 0.504 | 0.467 | 0.460 | 0.462 | 0.471 | 0.528 | 0.352 | 0.301 | 0.310 | | | | | | | | | |
| 12. ToLCV_Vellayani | 0.416 | 0.438 | 0.408 | 0.417 | 0.421 | 0.422 | 0.489 | 0.105 | 0.080 | 0.070 | 0.326 | | | | | | | | |
| 13. ToLCV_Neyyattinkara | 0.420 | 0.448 | 0.412 | 0.408 | 0.429 | 0.423 | 0.489 | 0.105 | 0.086 | 0.076 | 0.330 | 0.026 | | | | | | | |
| 14. ToLCV_Nedumangad | 0.433 | 0.418 | 0.411 | 0.412 | 0.418 | 0.433 | 0.442 | 0.329 | 0.280 | 0.293 | 0.296 | 0.300 | 0.313 | | | | | | |
| 15. ToLCV_Chirayinkeezhu | 0.437 | 0.435 | 0.417 | 0.426 | 0.432 | 0.432 | 0.458 | 0.324 | 0.298 | 0.307 | 0.308 | 0.322 | 0.316 | 0.064 | | | | | |
| 16. ToLCV_Kottarakkara | 0.439 | 0.425 | 0.403 | 0.433 | 0.424 | 0.429 | 0.450 | 0.435 | 0.392 | 0.394 | 0.372 | 0.403 | 0.405 | 0.351 | 0.330 | | | | |
| 17. ToLCV_Mukhathala | 0.478 | 0.461 | 0.455 | 0.473 | 0.464 | 0.474 | 0.486 | 0.353 | 0.318 | 0.332 | 0.326 | 0.330 | 0.336 | 0.221 | 0.228 | 0.324 | | | |
| 18. ToLCV_Punalur | 0.224 | 0.236 | 0.220 | 0.231 | 0.212 | 0.220 | 0.243 | 0.473 | 0.436 | 0.414 | 0.466 | 0.415 | 0.410 | 0.448 | 0.468 | 0.405 | 0.423 | | |
| 19. ToLCV_Tavanur | 0.224 | 0.236 | 0.220 | 0.231 | 0.212 | 0.220 | 0.243 | 0.473 | 0.436 | 0.414 | 0.466 | 0.415 | 0.410 | 0.448 | 0.468 | 0.405 | 0.423 | 0.000 | |

Fig. 4. Estimates of evolutionary divergence among different isolates of BYVMV, ToLCV and PYVMV isolates from Kerala.

location (Fig. 5). The details of BYVMV isolates selected for the comparison study were enlisted in Table 32. Similarly, the genetic distance analysis of BYVMV-Vellayani isolate with the selected BYVMV isolates from NCBI database revealed that, BYVMV-Vellayani expressed least divergence with other BYVMV isolate of Kerala while highest divergence to BYVMV-UP Varanasi isolate (Fig. 6).

In case of ToLCNDV-Vellayani isolate, the phylogenetic analysis of this isolate with other ToLCNDV isolates from NCBI database revealed that the virus isolate was more related to other ToLCNDV isolates from Thiruvananthapuram, Karnataka and Andhra Pradesh (Fig. 7); places that are of same geographical area. The details of BYVMV isolates selected for the comparison study were enlisted in Table 33. Further the genetic distance analysis revealed that ToLCNDV-Vellayani isolate expressed least genetic diversity to ToLCNDV isolate of Chithrakoot and highest diversity to ToLCGuV-Nepal isolate (Fig. 8).

When the phylogenetic analysis of SLCCNV isolate of Mukhathala was compared to homologous sequences from selected begomoviruses, it was found to be more related to SLCCNV isolates from Tamil Nadu and Gujarat (Fig. 9). The details of BYVMV isolates selected for the comparison study were enlisted in Table 34. While genetic distance analysis revealed least divergence of the virus to SLCCNV isolate of Coimbatore and maximum divergence to SLCuV isolate from Egypt (Fig. 10).

The phylogenetic analysis of the three viruses when carried out, revealed that ToLCNDV was more related to SLCCNV as compared to BYVMV (Fig. 11) though, all the viruses were clustered together confirming that they were related to each other.

## 4.8 DEVELOPING SPECIFIC PRIMERS FOR THE BEGOMOVIRUSES

Specific primers for each of the viruses *viz,*. BYVMV, ToLCNDV and SLCCNV were developed using Primer 3 software (http://bioinfo.ut.ee/primer3-

Table 32. List of BYVMV isolates selected from NCBI database for the phylogenetic study of BYVMV Vellayani

| Sl. No. | Name | Accession No. | Description | Location |
|---|---|---|---|---|
| 1 | BYVMV-PALAGHAT1 | GU112072.1 | *Bhendi yellow vein mosaic virus* [India:Phalaghat:OY138A:2006] segment DNA-A, complete sequence | India: Phalaghat, Kerala |
| 2 | BYVMV-PALAGHAT2 | GU112057.1 | *Bhendi yellow vein mosaic virus* [India:Kaivara:OYKaivara:2006] segment DNA-A, complete sequence | India: Kaivara, Karnataka |
| 3 | BYVMV-KERALA | GU112060.1 | *Bhendi yellow vein mosaic virus* [India:Kerala:OYG6AG:2005] segment DNA-A, complete sequence | India: Sonipat, Haryana |
| 4 | BYVMV-KARNATAKA | JQ326268.1 | *Bhendi yellow vein mosaic virus* isolate OY173 segment DNA-A, complete sequence | India: Pandarahalli, Tamil Nadu |
| 5 | BYVMV-MADURAI | AF241479.1 | *Bhendi yellow vein mosaic virus*-[Madurai] segment A, complete sequence | India |
| 6 | BYVMV-COIMBATORE | GU112080.1 | *Bhendi yellow vein mosaic virus* [India:Coimbator:OYCO4:2005] segment DNA-A, complete sequence | India: Coimbator, Tamil Nadu |
| 7 | BYVMV-LUCKNOW | KC501921.1 | *Bhendi yellow vein mosaic virus* isolate EL26 segment DNA-A, complete sequence | India: Lucknow, Uttar Pradesh |
| 8 | BYVMV-THAILAND1 | JX678967.1 | *Bhendi yellow vein mosaic virus* isolate WTHOK6, complete genome | Thailand: Nakhon Pathom |

| | | | | |
|---|---|---|---|---|
| 9 | BYVMV-THAILAND2 | JX678966.1 | *Bhendi yellow vein mosaic virus* isolate Ok2, complete genome | Thailand: Kanchanaburi |
| 10 | BYVMV-MAHARASHTRA | JQ359505.1 | *Bhendi yellow vein mosaic virus* isolate 126B segment DNA-A, complete sequence | India: Jalaon, Maharasthra |
| 11 | BYVMV-HIMACHAL | FR694925.1 | *Bhendi yellow vein mosaic virus* complete sequence, isolate Himachal | India:Palampur, Himachal Pradesh |
| 12 | BYVMV-PAKISTAN1 | MH748679.1 | *Bhendi yellow vein mosaic virus* clone ssr29 segment DNA-A, complete sequence | Pakistan |
| 13 | BYVMV-SRILANKA1 | MH455212.1 | *Bhendi yellow vein mosaic virus* isolate Ma02, complete genome | Sri Lanka:Matara |
| 14 | BYVMV-SRILANKA2 | MH455211.1 | *Bhendi yellow vein mosaic virus* isolate Pu02, complete genome | Sri Lanka:Puttalam |
| 15 | BYVMV-UP VARANASI | KT390451.1 | *Bhendi yellow vein mosaic virus* isolate WOK3 segment DNA A, complete sequence | India: Uttar Pradesh, Varanasi |
| 16 | OELCV-SRILANKA1 | KX698093.1 | *Okra enation leaf curl virus* isolate Batticaloa segment DNA-A, complete sequence | Sri Lanka |
| 17 | OELCV-SRILANKA2 | KX698092.1 | *Okra enation leaf curl virus* isolate Matara segment DNA-A, complete sequence | Sri Lanka |
| 18 | OELCV-SRILANKA3 | KX698091.1 | *Okra enation leaf curl virus* isolate Puttalam segment DNA-A, complete sequence | Sri Lanka |

| | | | | |
|---|---|---|---|---|
| 19 | BYVMV-RAJASTAN | KT390321.1 | *Bhendi yellow vein mosaic virus* isolate OK303-RAJ segment DNA A, complete sequence | India: Rajasthan, Udaipur |
| 20 | BYVMV-PAKISTAN2 | AJ002451.1 | *Okra yellow vein mosaic virus*-[201], segment A, complete sequence | Pakistan |

Table 33. List of ToLCNDV isolates selected from NCBI database for the phylogenetic study of ToLCNDV Vellayani

| Sl. No. | Name of virus | Description | Acc. No. | Location |
|---|---|---|---|---|
| 1 | ToLCNDV-ANDHRA PRADESH | *Tomato leaf curl New Delhi virus* isolate TC89 segment DNA-A, complete sequence | KP195265.1 | India, Andhra Pradesh |
| 2 | ToLCNDV-TRIVANDRUM | *Tomato leaf curl New Delhi virus* isolate RG5, Complete genome | KT426907.1 | India, Kerala, Trivandrum |
| 3 | ToLCNDV-PALAGHAT | *Tomato leaf curl New Delhi virus* isolate BG1 segment DNA A, complete sequence | KP868764.1 | India: Phalaghat, Kerala |
| 4 | ToLCKeV-PAKISTAN1 | *Tomato leaf curl Kerala virus* isolate RM430, complete genome | KY926898.1 | Pakistan |
| 5 | ToLCPatV-BANGLADESH | *Tomato leaf Curl Patna virus*-[Bangladesh:Jamalpur: 2014] isolate ToLCV-JB segment DNA-A, complete sequence | KU933675.1 | Bangladesh: Joydebpur |
| 6 | ToLCKeV – PAKISTAN2 | *Tomato leaf curl Kerala virus* isolate begomovirus genome assembly, segment: I | LT556075.1 | Pakistan |
| 7 | ToLCGUV-NEPAL | *Tomato leaf curl Gujarat virus* - [Nepal] segment DNA-A, complete sequence | AY234383.1 | Nepal,Panchkhal |
| 8 | ToLCNDV-TAMILNADU | *Tomato leaf curl New Delhi virus* isolate CI1 segment DNA A, complete sequence | KY979591.1 | India: Tamil Nadu |
| 9 | ToLCNDV-SPAIN | *Tomato leaf curl New Delhi virus* isolate tomato-A4 segment DNA-A, complete sequence | KM977733.1 | Spain: Almeria |
| 10 | ToLCNDV-DELHI | *Tomato leaf curl New Delhi virus* isolate India:Delhi:Cucumis:2012 segment DNA-A, complete sequence | KC545812.1 | India: New Delhi |
| 11 | ToLCNDV–MAHARASHTRA | *Tomato leaf curl New Delhi virus* isolate eggplant | HQ264185.1 | India: Maharastra |

| | | | | |
|---|---|---|---|---|
| | | segment DNA-A, complete sequence | | |
| 12 | ToLCNDV-TUNISIA | *Tomato leaf curl New Delhi virus* isolate D22 segment DNA-A, complete sequence | MF967020.1 | Tunisia |
| 13 | ToLCNDV-COMBODIA | *Tomato leaf curl New Delhi virus* isolate KH_winter melon_Q6568 segment DNA-A, complete sequence | MH328257.1 | Cambodia |
| 14 | ToLCNDV-LAOS | *Tomato leaf curl New Delhi virus* isolate LA_M.charantia_Q6419 segment DNA-A, complete sequence | MH328255.1 | Laos |
| 15 | ToLCNDV–BANGALORE | *Tomato leaf curl New Delhi virus* isolate PV234 segment DNA-A, complete sequence | MK336424.1 | India: Bangalore |
| 16 | ToLCNDV-UP VARANASI | *Tomato leaf curl New Delhi virus* isolate SPYG1 segment DNA-A, complete sequence | KY780213.1 | India: Varanasi, Utter Pradesh |
| 17 | ToLCNDV-BIHAR | *Tomato leaf curl New Delhi virus* isolate ToLCNDV-SBO (SN) segment DNA-A, complete sequence | MH465599.1 | India |
| 18 | ToLCNDV-SEYCHELLES | *Tomato leaf curl New Delhi virus* isolate Seychelles-Praslin-SC8-2017 segment DNA-A, complete sequence | MH511991.1 | Seychelles |
| 19 | ToLCNDV-CHITRAKOOT | *Tomato leaf curl New Delhi virus* isolate India:ToLCNDV-CKTD:2017 segment DNA-A, complete sequence | MF807949.1 | India: Chitrakoot |
| 20 | ToLCNDV -MOROCCO | *Tomato leaf curl New Delhi virus* isolate Agadir segment DNA A, complete sequence | MG098230.1 | Morocco |

Table 34. List of SLCCNV isolates selected from NCBI database for the phylogenetic study of SLCCNV Mukhathala

| Sl. No. | Name | Accession No. | Description | Location |
|---|---|---|---|---|
| 1. | SLCCNV-COIMBATORE | AY184487.3 | *Squash leaf curl China virus* - [Pumpkin :Coimbatore] segment DNA-A, complete sequence | Coimbatore |
| 2. | SLCCNV-GUJRAT | KF188433.1 | *Squash leaf curl China virus* isolate KP1 segment DNA-A, complete sequence | India |
| 3. | SLCCNV-UTHAR PRADESH 1 | DQ026296.2 | *Squash leaf curl China virus* - [Pumpkin: Lucknow] segment DNA A, complete sequence | Lucknow |
| 4. | SLCCNV-UTHAR PRADESH 2 | MH836313.1 | *Squash leaf curl China virus* isolate Sq-1 segment DNA-A, complete sequence | India:Utter Pradesh,Varanasi |
| 5. | SLCCNV-UTHAR PRADESH 3 | MH816957.1 | *Squash leaf curl China virus* isolate PG1 segment DNA-B, complete sequence | India:Utter Pradesh,Varanasi |
| 6. | SLCCNV-CHINA 1 | MG525551.1 | *Squash leaf curl China virus* isolate Guangxi2017 segment DNA-A, complete sequence | China |
| 7. | SLCCNV-VIETNAM | KC857509.1 | *Squash leaf curl China virus* isolate Hanoi segment DNA-A, complete sequence | Viet Nam |
| 8. | SLCCNV–CHINA 2 | KC171648.1 | *Squash leaf curl China virus* isolate GZ01 segment DNA-A, complete sequence | China |
| 9. | SLCCNV –CHINA 3 | MF062251.1 | *Squash leaf curl China virus* isolate Hn segment DNA-A, complete | China |

| | | | | |
|---|---|---|---|---|
| | | | sequence | |
| 10. | SLCCNV-TAMIL NADU | MF102264.1 | *Squash leaf curl China virus* isolate J1 segment DNA-A, complete sequence | India: Tamilnadu |
| 11. | SLCCNV –CHINA 4 | KF999983.1 | *Squash leaf curl China virus* isolate SY segment DNA-A, complete sequence | China: Hainan |
| 12. | SLCCNV –CHINA 5 | KF184993.1 | *Squash leaf curl China virus* isolate HA3 segment DNA-A, complete sequence | China |
| 13. | SLCCNV –CHINA 6 | KF184992.1 | *Squash leaf curl China virus* isolate HA1 segment DNA-A, complete sequence | China |
| 14. | SLCCNV-NEW DELHI | JN587811.1 | *Squash leaf curl China virus* isolate SLCCNV-[Pum:IARI] segment DNA-A, complete sequence | India |
| 15. | SLCCNV-PHILIPPINES | EU487031.1 | *Squash leaf curl China virus* isolate P54, complete genome | Philippines: Benguet |
| 16. | SLCuV-PALESTINE | KC441465.1 | *Squash leaf curl virus* isolate PAL segment DNA-A AV1, AC3, AC2, AC1, and AC4 genes, complete cds | Palestine |
| 17. | SLCuV-TAIWAN | EU479710.1 | *Squash leaf curl virus* isolate YL segment DNA A, complete sequence | Taiwan: Yunlin |
| 18. | SLCuV-EGYPT | MG763920.1 | *Squash leaf curl virus* isolate Kaha segment DNA-A, complete sequence | Egypt |

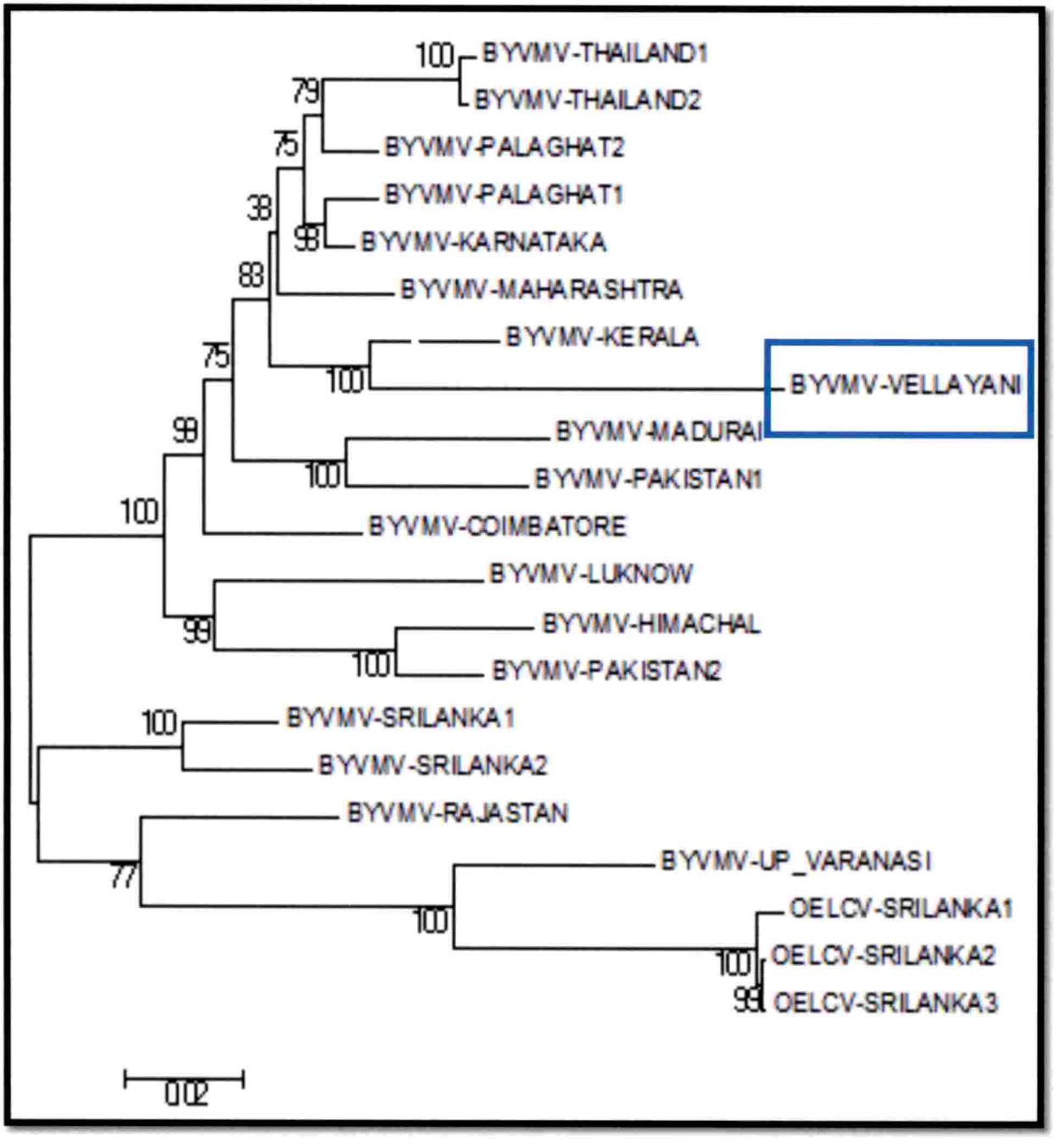


Fig 5. Phylogenetic tree of the complete nucleotide sequence of the DNA-A component of BYVMV Vellayani isolate with selected BYVMV isolates from India and other countries retrieved from NCBI database. Phylogenetic tree was constructed using the neighbor-joining method with the MEGA 6 software program. Bootstrap scores in percent were depicted at nodes (500 replicates).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. BYVMV-PALAGHAT1 | | | | | | | | | | | | | | | | | | | | | |
| 2. BYVMV-PALAGHAT2 | 0.023 | | | | | | | | | | | | | | | | | | | | |
| 3. BYVMV-KERALA | 0.058 | 0.047 | | | | | | | | | | | | | | | | | | | |
| 4. BYVMV-KARNATAKA | 0.013 | 0.020 | 0.054 | | | | | | | | | | | | | | | | | | |
| 5. BYVMV-MADURAI | 0.078 | 0.072 | 0.085 | 0.077 | | | | | | | | | | | | | | | | | |
| 6. BYVMV-COIMBATORE | 0.054 | 0.055 | 0.087 | 0.049 | 0.094 | | | | | | | | | | | | | | | | |
| 7. BYVMV-LUKNOW | 0.086 | 0.091 | 0.107 | 0.081 | 0.122 | 0.101 | | | | | | | | | | | | | | | |
| 8. BYVMV-THAILAND1 | 0.042 | 0.034 | 0.066 | 0.038 | 0.093 | 0.054 | 0.100 | | | | | | | | | | | | | | |
| 9. BYVMV-THAILAND2 | 0.041 | 0.033 | 0.064 | 0.037 | 0.092 | 0.052 | 0.098 | 0.004 | | | | | | | | | | | | | |
| 10. BYVMV-MAHARASHTRA | 0.033 | 0.039 | 0.062 | 0.029 | 0.068 | 0.065 | 0.084 | 0.056 | 0.055 | | | | | | | | | | | | |
| 11. BYVMV-HIMACHAL | 0.104 | 0.102 | 0.113 | 0.102 | 0.124 | 0.089 | 0.089 | 0.119 | 0.117 | 0.104 | | | | | | | | | | | |
| 12. BYVMV-PAKISTAN1 | 0.078 | 0.078 | 0.091 | 0.077 | 0.064 | 0.081 | 0.123 | 0.095 | 0.093 | 0.072 | 0.104 | | | | | | | | | | |
| 13. BYVMV-SRILANKA1 | 0.105 | 0.103 | 0.135 | 0.103 | 0.130 | 0.074 | 0.120 | 0.099 | 0.097 | 0.109 | 0.120 | 0.102 | | | | | | | | | |
| 14. BYVMV-SRILANKA2 | 0.109 | 0.109 | 0.143 | 0.107 | 0.137 | 0.086 | 0.129 | 0.113 | 0.112 | 0.115 | 0.126 | 0.111 | 0.037 | | | | | | | | |
| 15. BYVMV-UP_VARANASI | 0.182 | 0.184 | 0.160 | 0.180 | 0.177 | 0.176 | 0.175 | 0.187 | 0.185 | 0.180 | 0.170 | 0.160 | 0.128 | 0.123 | | | | | | | |
| 16. OELCV-SRILANKA1 | 0.208 | 0.209 | 0.181 | 0.208 | 0.215 | 0.197 | 0.201 | 0.208 | 0.208 | 0.201 | 0.201 | 0.184 | 0.141 | 0.125 | 0.089 | | | | | | |
| 17. OELCV-SRILANKA2 | 0.205 | 0.207 | 0.178 | 0.205 | 0.212 | 0.194 | 0.198 | 0.205 | 0.205 | 0.198 | 0.197 | 0.181 | 0.138 | 0.123 | 0.085 | 0.006 | | | | | |
| 18. OELCV-SRILANKA3 | 0.205 | 0.207 | 0.178 | 0.205 | 0.212 | 0.194 | 0.199 | 0.205 | 0.205 | 0.198 | 0.198 | 0.181 | 0.138 | 0.122 | 0.085 | 0.006 | 0.001 | | | | |
| 19. BYVMV-RAJASTAN | 0.101 | 0.102 | 0.134 | 0.099 | 0.130 | 0.112 | 0.134 | 0.106 | 0.105 | 0.110 | 0.145 | 0.133 | 0.120 | 0.119 | 0.110 | 0.148 | 0.144 | 0.144 | | | |
| 20. BYVMV-PAKISTAN2 | 0.095 | 0.094 | 0.102 | 0.092 | 0.109 | 0.078 | 0.098 | 0.113 | 0.111 | 0.093 | 0.038 | 0.094 | 0.113 | 0.118 | 0.163 | 0.191 | 0.189 | 0.189 | 0.134 | | |
| 21. BYVMV-VELLAYANI | 0.102 | 0.106 | 0.091 | 0.096 | 0.143 | 0.134 | 0.147 | 0.123 | 0.123 | 0.105 | 0.165 | 0.139 | 0.174 | 0.171 | 0.206 | 0.182 | 0.179 | 0.179 | 0.178 | 0.155 | |

Fig. 6. Estimates of evolutionary divergence of BYVMV Vellayani isolate with selected BYVMV isolates from India and other countries retrieved from NCBI database.

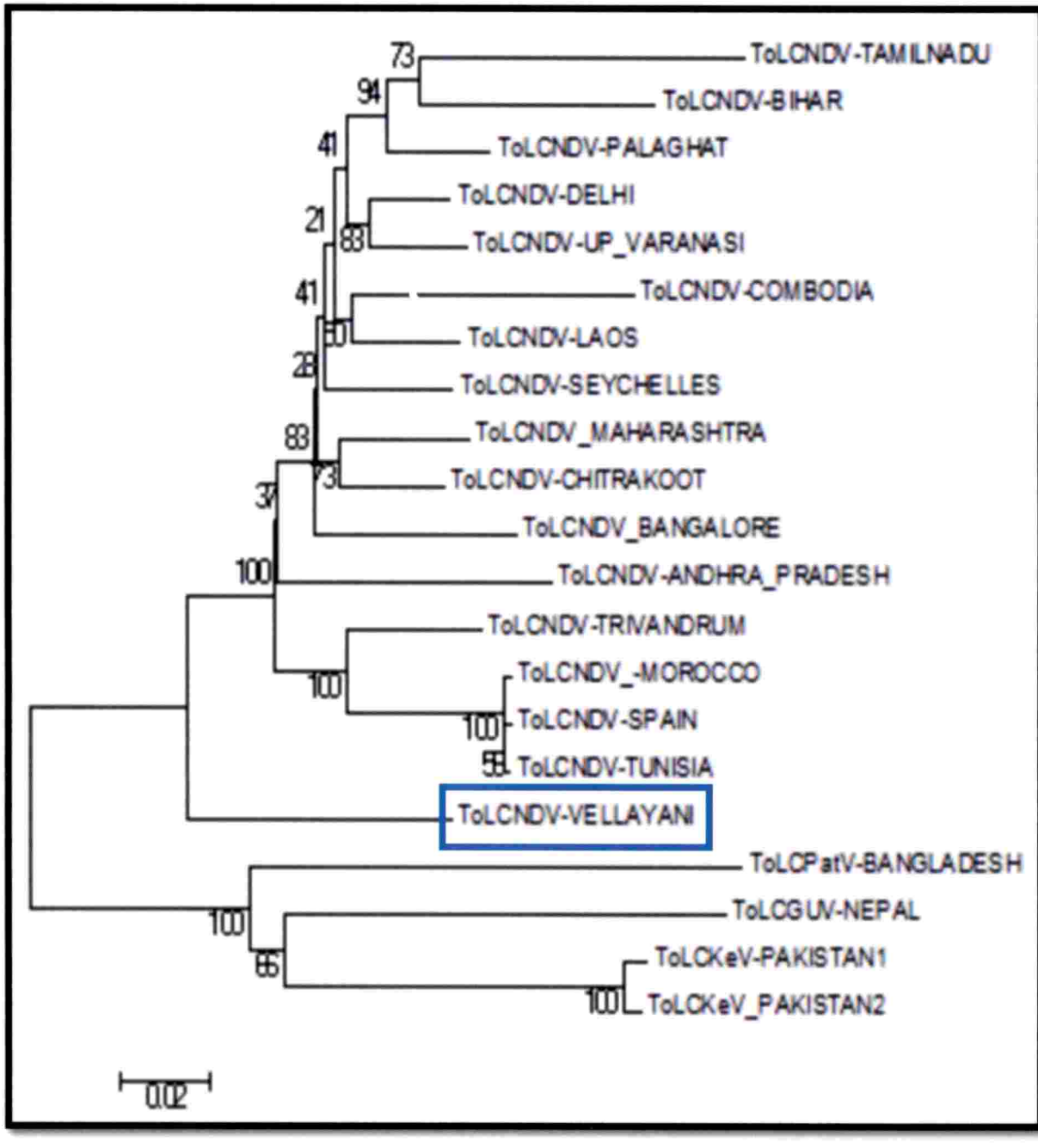


Fig 7. Phylogenetic tree of the complete nucleotide sequence of the DNA-A component of ToLCNDV Vellayani isolate with selected ToLCV isolates retrieved from NCBI database belonging to India and other countries. Constructed using the neighbor-joining method with the MEGA 6 software program. Bootstrap scores in percent were depicted at nodes (500 replicates).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. ToLCNDV-ANDHRA_PRADESH | | | | | | | | | | | | | | | | | | | | | |
| 2. ToLCNDV-TRIVANDRUM | 0.111 | | | | | | | | | | | | | | | | | | | | |
| 3. ToLCNDV-PALAGHAT | 0.106 | 0.089 | | | | | | | | | | | | | | | | | | | |
| 4. ToLCKeV-PAKISTAN1 | 0.254 | 0.242 | 0.247 | | | | | | | | | | | | | | | | | | |
| 5. ToLCPatV-BANGLADESH | 0.269 | 0.265 | 0.260 | 0.189 | | | | | | | | | | | | | | | | | |
| 6. ToLCKeV_PAKISTAN2 | 0.253 | 0.241 | 0.246 | 0.009 | 0.188 | | | | | | | | | | | | | | | | |
| 7. ToLCGUV-NEPAL | 0.263 | 0.263 | 0.257 | 0.179 | 0.227 | 0.179 | | | | | | | | | | | | | | | |
| 8. ToLCNDV-TAMILNADU | 0.169 | 0.152 | 0.093 | 0.274 | 0.292 | 0.272 | 0.287 | | | | | | | | | | | | | | |
| 9. ToLCNDV-SPAIN | 0.109 | 0.066 | 0.096 | 0.241 | 0.263 | 0.239 | 0.263 | 0.160 | | | | | | | | | | | | | |
| 10. ToLCNDV-DELHI | 0.099 | 0.086 | 0.058 | 0.247 | 0.262 | 0.244 | 0.256 | 0.116 | 0.093 | | | | | | | | | | | | |
| 11. ToLCNDV_MAHARASHTRA | 0.104 | 0.088 | 0.071 | 0.244 | 0.263 | 0.243 | 0.261 | 0.133 | 0.097 | 0.060 | | | | | | | | | | | |
| 12. ToLCNDV-TUNISIA | 0.110 | 0.066 | 0.096 | 0.240 | 0.263 | 0.238 | 0.262 | 0.159 | 0.003 | 0.093 | 0.097 | | | | | | | | | | |
| 13. ToLCNDV-COMBODIA | 0.135 | 0.122 | 0.104 | 0.248 | 0.264 | 0.245 | 0.258 | 0.143 | 0.124 | 0.097 | 0.104 | 0.124 | | | | | | | | | |
| 14. ToLCNDV-LAOS | 0.102 | 0.083 | 0.060 | 0.248 | 0.263 | 0.246 | 0.264 | 0.119 | 0.099 | 0.053 | 0.064 | 0.098 | 0.087 | | | | | | | | |
| 15. ToLCNDV_BANGALORE | 0.116 | 0.092 | 0.086 | 0.256 | 0.269 | 0.254 | 0.268 | 0.147 | 0.103 | 0.076 | 0.085 | 0.103 | 0.118 | 0.076 | | | | | | | |
| 16. ToLCNDV-UP_VARANASI | 0.106 | 0.093 | 0.054 | 0.246 | 0.263 | 0.243 | 0.256 | 0.116 | 0.102 | 0.040 | 0.066 | 0.102 | 0.104 | 0.058 | 0.084 | | | | | | |
| 17. ToLCNDV-BIHAR | 0.141 | 0.131 | 0.093 | 0.253 | 0.277 | 0.252 | 0.250 | 0.125 | 0.134 | 0.090 | 0.108 | 0.133 | 0.125 | 0.106 | 0.129 | 0.097 | | | | | |
| 18. ToLCNDV-SEYCHELLES | 0.095 | 0.091 | 0.065 | 0.249 | 0.265 | 0.246 | 0.259 | 0.125 | 0.098 | 0.056 | 0.063 | 0.098 | 0.102 | 0.058 | 0.076 | 0.048 | 0.105 | | | | |
| 19. ToLCNDV-CHITRAKOOT | 0.101 | 0.081 | 0.065 | 0.245 | 0.266 | 0.244 | 0.260 | 0.127 | 0.090 | 0.057 | 0.052 | 0.089 | 0.103 | 0.064 | 0.069 | 0.059 | 0.102 | 0.059 | | | |
| 20. ToLCNDV -MOROCCO | 0.111 | 0.067 | 0.096 | 0.238 | 0.262 | 0.237 | 0.261 | 0.159 | 0.004 | 0.093 | 0.098 | 0.004 | 0.125 | 0.099 | 0.102 | 0.103 | 0.134 | 0.099 | 0.090 | | |
| 21. ToLCNDV-VELLAYANI | 0.146 | 0.125 | 0.125 | 0.207 | 0.260 | 0.206 | 0.262 | 0.184 | 0.131 | 0.122 | 0.113 | 0.130 | 0.152 | 0.117 | 0.134 | 0.122 | 0.163 | 0.124 | 0.101 | 0.131 | |

Fig. 8. Estimates of evolutionary divergence of ToLCNDV Vellayani isolate with other isolates of ToLCV isolates from India and other countries retrieved from NCBI database.

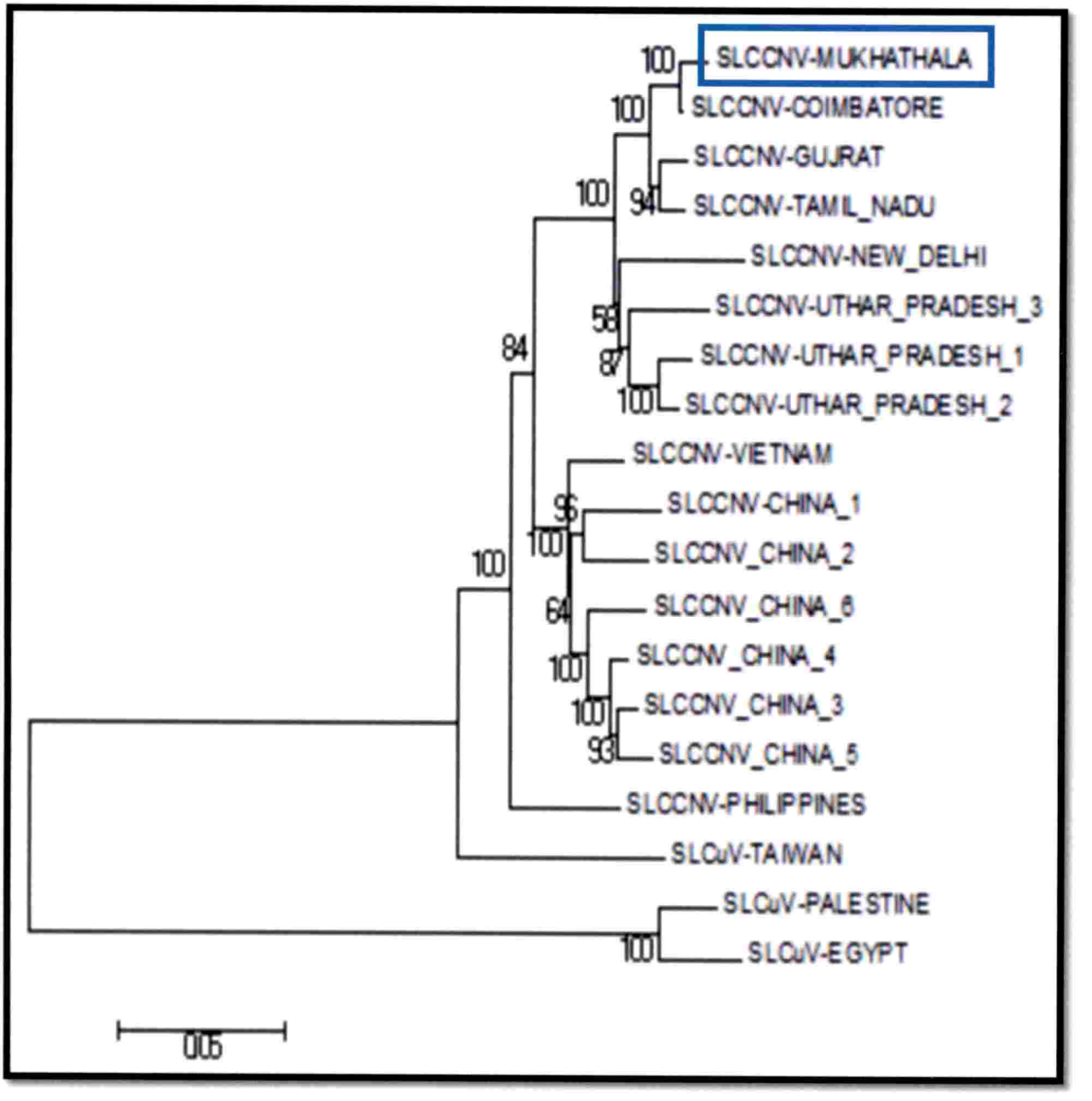


Fig 9. Phylogenetic tree of the complete nucleotide sequence of the DNA-A component of SLCCNV Vellayani isolate with selected SLCV isolates retrieved from NCBI database belonging to India and other countries. Constructed using the neighbor-joining method with the MEGA 6 software program. Bootstrap scores in percent were depicted at nodes (500 replicates).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. SLCCNV-MUKHATHALA | | | | | | | | | | | | | | | | | | | |
| 2. SLCCNV-COIMBATORE | 0.010 | | | | | | | | | | | | | | | | | | |
| 3. SLCCNV-GUJRAT | 0.028 | 0.020 | | | | | | | | | | | | | | | | | |
| 4. SLCCNV-UTHAR_PRADESH_1 | 0.047 | 0.039 | 0.042 | | | | | | | | | | | | | | | | |
| 5. SLCCNV-UTHAR_PRADESH_2 | 0.045 | 0.036 | 0.040 | 0.016 | | | | | | | | | | | | | | | |
| 6. SLCCNV-UTHAR_PRADESH_3 | 0.057 | 0.049 | 0.051 | 0.044 | 0.037 | | | | | | | | | | | | | | |
| 7. SLCCNV-CHINA_1 | 0.092 | 0.084 | 0.082 | 0.085 | 0.082 | 0.090 | | | | | | | | | | | | | |
| 8. SLCCNV-VIETNAM | 0.079 | 0.072 | 0.071 | 0.075 | 0.070 | 0.081 | 0.045 | | | | | | | | | | | | |
| 9. SLCCNV_CHINA_2 | 0.086 | 0.079 | 0.078 | 0.082 | 0.075 | 0.085 | 0.043 | 0.041 | | | | | | | | | | | |
| 10. SLCCNV_CHINA_3 | 0.085 | 0.078 | 0.078 | 0.082 | 0.077 | 0.086 | 0.045 | 0.037 | 0.045 | | | | | | | | | | |
| 11. SLCCNV-TAMIL_NADU | 0.030 | 0.022 | 0.015 | 0.043 | 0.040 | 0.048 | 0.083 | 0.072 | 0.079 | 0.078 | | | | | | | | | |
| 12. SLCCNV_CHINA_4 | 0.084 | 0.076 | 0.076 | 0.080 | 0.076 | 0.086 | 0.042 | 0.035 | 0.040 | 0.013 | 0.075 | | | | | | | | |
| 13. SLCCNV_CHINA_5 | 0.088 | 0.081 | 0.082 | 0.085 | 0.081 | 0.091 | 0.050 | 0.042 | 0.048 | 0.017 | 0.082 | 0.018 | | | | | | | |
| 14. SLCCNV_CHINA_6 | 0.085 | 0.078 | 0.079 | 0.082 | 0.078 | 0.085 | 0.052 | 0.041 | 0.044 | 0.032 | 0.079 | 0.030 | 0.036 | | | | | | |
| 15. SLCCNV-NEW_DELHI | 0.066 | 0.057 | 0.066 | 0.058 | 0.054 | 0.065 | 0.102 | 0.093 | 0.100 | 0.093 | 0.061 | 0.095 | 0.100 | 0.097 | | | | | |
| 16. SLCCNV-PHILIPPINES | 0.098 | 0.092 | 0.093 | 0.093 | 0.089 | 0.096 | 0.078 | 0.063 | 0.073 | 0.071 | 0.092 | 0.068 | 0.075 | 0.074 | 0.109 | | | | |
| 17. SLCuV-PALESTINE | 0.401 | 0.396 | 0.396 | 0.395 | 0.394 | 0.395 | 0.398 | 0.394 | 0.400 | 0.397 | 0.394 | 0.394 | 0.394 | 0.392 | 0.397 | 0.393 | | | |
| 18. SLCuV-TAIWAN | 0.137 | 0.130 | 0.133 | 0.132 | 0.127 | 0.138 | 0.118 | 0.109 | 0.118 | 0.109 | 0.130 | 0.112 | 0.117 | 0.117 | 0.145 | 0.102 | 0.397 | | |
| 19. SLCuV-EGYPT | 0.408 | 0.403 | 0.403 | 0.402 | 0.401 | 0.402 | 0.406 | 0.402 | 0.408 | 0.404 | 0.401 | 0.402 | 0.402 | 0.399 | 0.403 | 0.400 | 0.042 | 0.405 | |

Fig. 10. Estimates of evolutionary divergence of SLCCNV Mukhathala isolate with other isolates of SLCCNV isolates from India and other countries retrieved from NCBI database.

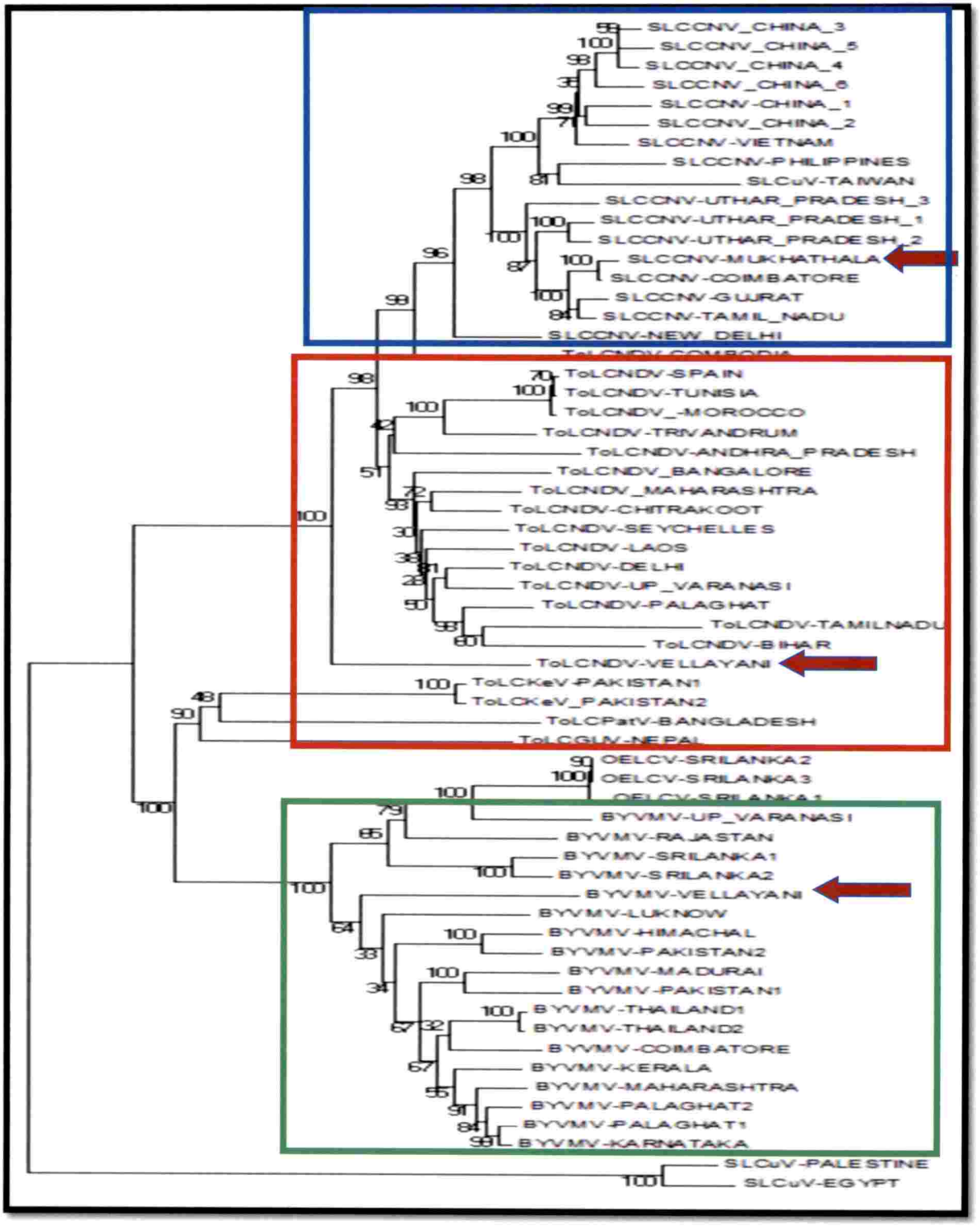


Fig 11. Phylogenetic tree of the complete nucleotide sequence of the DNA-A component of BYVMV-Vellayani, ToLCNDV-Vellayani and SLCCNV-Mukhathala with selected virus isolates from NCBI database constructed using the neighbor-joining method with the MEGA 6 software program.

0.4.0/). For the primer development, the overall variability among selected isolates of each of the viruses were done. In case of BYVMV highest variability was observed in AC4 ORF region while in ToLCNDV the variability was observed in AC2 ORF region; whereas in SLCCNV in AV2 ORF region (Table 35). The primers designed with the sequence, the ORF from which it was designed and the products size are mentioned in Table 36.

Table 35. Overall mean divergence (d) of different open reading frames of BYVVMV, ToLCNDV and SLCCNV isolates based on aminoacid sequences

| Virus | Open reading frame (ORF) | | | | | | |
|---|---|---|---|---|---|---|---|
| | AV2 | AV1 | AC5 | AC3 | AC2 | AC1 | AC4 |
| BYVMV | 0.097* ± 0.014 | 0.090 ± 0.009 | 0.120 ± 0.020 | 0.083 ± 0.014 | 0.079 ± 0.019 | 0.120 ± 0.010 | 0.124 ± 0.026 |
| ToLCNDV | 0.180 ± 0.022 | 0.080 ± 0.007 | 0.232 ± 0.025 | 0.221 ± 0.020 | 0.241 ± 0.020 | 0.082 ± 0.008 | 0.222 ± 0.031 |
| SLCCNV | 0.108 ± 0.019 | 0.024 ± 0.007 | 0.084 ± 0.016 | 0.079 ± 0.013 | 0.052 ± 0.007 | 0.092 ± 0.028 | |

Table 36. List of specific primers designed for detection of BYVMV, ToLCNDV and SLCCNV

| Virus | Primer | Sequence (5'-3') | Region/ORF | Product size |
|---|---|---|---|---|
| BYVMV | B (F) | TCTGAAACTCTCCCCATTCG | AC4 | 327 bp |
| | B (R) | TCCAGTTCGAGGGCAAATAC | | |
| ToLCNDV | T (F) | CTCTCAAGAAACGCCCAGTC | AC2 (Transcriptional activator protein) | 454 bp |
| | T (R) | ACGACGAGAGACCTTGCCTA | | |
| SLCCNV | S (F) | CCTGAAAGCGTTCATGGTCT | AV2 | 350 bp |
| | S (R) | ATAGGGGCTGTCGAAGTTGA | | |

# *Discussion*

# 5. DISCUSSION

The objective of this study was "Immunomolecular detection and genetic analysis of the begomoviruses *viz., Bhendi yellow vein mosaic virus*, *Tomato leaf curl virus* and *Pumpkin yellow vein mosaic virus*". In view of evolution of variants of Begomovirus family of plant viruses, it becomes imperative to characterize the diseased plants for the identification of isolates of the viruses causing infection. Information about sequence diversity and biodiversity was generated to improve knowledge about this important group of viruses. The present work describes the results of molecular study of the sequences of genomes of different BYVMV, ToLCV and PYVMV isolates collected from the Kerala in an effort towards identifying and determining the molecular variability of the three begomoviruses. Discussion of the results obtained is presented in this chapter.

## 5.1 COLLECTION OF BEGOMOVIRUS INFECTED OKRA, TOMATO AND PUMPKIN FROM THIRUVANANTHAPURAM AND KOLLAM DISTRICTS

In the first part of this study, symptomatic okra, tomato and pumpkin crop samples were collected from three different sites in two districts of Kerala *viz.,* Thiruvananthapuram and Kollam. The study on the disease incidence and vulnerability index of the infected crops revealed that most of the fields surveyed exhibited cent per cent disease incidence and more than 50.00 as the V.I. value. It might be due to the facts that the surveys were done at the late stage of infection of the crop and survey conducted coincided with summer season, when the vector population was high. Similar observations were also reported by Ghevariya and Mahatma (2017) where the heavy incidence of BYVMD in the different fields of Gujarat was recorded during late winter to summer of the survey conducted in 2015. A survey on begomoviruses associated with okra also revealed that YVMV incidence in Kerala range from 42.45 to 75.64 per cent (Venkataravanappa, 2008). Anandhan *et*

*al.* (2011) also noticed a similar trend in D.I. wherein ToLCD incidence was higher in the month of October (73.7 %) than February (49.8 %) in all the reported years. Saklani and Mathai (1977) observed that D.I. was 35 % and 69 % in winter and summer, respectively. The observations of this study were thus in accordance to that of Muniyappa *et al.* (1991, 2000) that in summer, generally cent per cent infection of ToLCD was observed with 27 to 90 per cent crop loss.

Bhagabati and Goswami (1992) observed that whitefly populations were highest in crops sown in May and the incidence of *Bhendi yellow vein mosaic virus* was highest (100 %) in crops sown in May and June. A high positive correlation was observed between disease incidence and the population of *B. tabaci*. Nath *et al.* (1992) revealed that the incidence of the disease caused by *Okra yellow vein mosaic virus* was lowest, when populations of the vector, *B. tabaci*, were low. Significant positive associations were recorded between disease incidence and whitefly population. Similar results were also observed by several workers (Burger *et al.*, 1988; Pun *et al.*, 2005; Magar and Nirmal, 2010).

The variation in the D.I. and severity depended on many factors such as survival of viruliferous whiteflies and their biotypes, availability of alternative hosts and volunteer plants grown in the farm houses during off-season for survival of both the virus and vector, cultural operations including all management practices followed by farmers, host plant resistance, etc. as was put forth by Anandhan *et al.* (2011).

## 5.2 SYMPTOMATOLOGY

The preliminary requirement for management of any disease was the identification of the associated pathogen through the symptoms that expressed in the host plants. Hence, symptomatology was a prerequisite for understanding the pathogen, in this case the virus involved.

Symptoms expressed by *Bhendi yellow vein mosaic virus* (BYVMV) infecting okra plants varied depending on the stage of infection. The symptoms observed from all the locations were similar with vein clearing as the early symptom. As the disease progressed the whole leaf turned chlorotic which could be due to the degradation and reduction of chlorophyll content as suggested by Uppal *et al.* (1940). Chlorosis was accompanied by blistering and distortion of leaf, reduced size of leaf, vein thickening, stunted growth, reduced flowering and fruiting, vein clearing of flower buds and malformed small sized fruits. A similar observation was recorded by Capoor and Varma (1950) with characterized vein clearing, chlorosis, swelling coupled with slight downward curling of leaf margins, twisting of petioles, and retardation of growth in the virus infected bhindi plants .

Tomato plants infected with ToLCD expressed symptoms like leaf curling, distorted small sized leaves, stunted growth of plant and reduced flowering and fruiting which were in accordance with the observations of Anandhan *et al.* (2011). Similarly, symptoms like inward rolling of the leaf margins, puckering, vein thickening/enation, shortening of internodal length and bushy appearance in addition to mosaic pattern on the leaves, excessive branching and small-sized leaves were also recorded from different locations of India by other scientists (Vasudeva and Samraj, 1948; Singh, 1989; Pandey *et al.*, 2010; Kumari *et al.*, 2011). The additional symptoms of ToLCV infection such as purplish discoloration of leaves, leaf cupping, yellowing and mottling of leaf lamina and virescence of flowers from Vellayani and Neyyattinkara of Thiruvananthapuram could be due to the fact that ToLCNDV was associated with the ToLCD in these areas. The symptoms were similar to those recorded by Pandey *et al.* (2010) on tomato seedlings infected with ToLCNDV, which further confirmed the above observation.

Symptomatology of *Pumpkin yellow vein mosaic* (PYVMV) infected pumpkin plants included initial vein clearing followed by mosaic of leaf lamina. In later stage of infection, the leaves became completely chlorotic along with reduction in leaf size,

stunted growth of plant, reduction in flowering and fruiting, and vein clearing on floral parts. Muniyappa *et al.* (2003) had recorded similar symptoms in PYVMD infected pumpkin plants such as initial vein clearing followed by mosaic, retarded plant growth, senescence of flowers, reduced fruit set and shriveled and under-sized fruits.

## 5.3 TRANSMISSION STUDIES

Modes of transmission of viruses was another factor of prime importance since the disease incidence and subsequent economic loss due to high D.I. lies upon this one major factor. Hence, better understanding of the different modes of transmission of the three begomoviruses were of utmost importance. In the present investigation, the transmission study of the viruses were done by insect, graft and seed transmission.

### 5.3.1 Insect transmission

Muniyappa (1980) found that only known method of transmission of BYVMV was through whitefly (*Bemisia tabaci* Genn.). In this study, the whitefly transmission of BYVMV, ToLCV and PYVMV in one-week old seedlings of okra, tomato and pumpkin revealed that three weeks (20 days) after inoculation all the three crops expressed initial symptoms of the diseases. A similar observation was recorded by Anandhan *et al.* (2011) and it took 16-18 days for symptom expression for tomato seedlings inoculated with ToLCV. In contrast to the above observations, Muniyappa *et al.* (2003) recorded an early symptom expression in pumpkin seedlings inoculated with PYVMV after 8 days of inoculation. Though Venkataravanappa *et al.* (2013b) reported that 15 DAI okra seedlings expressed YVMD symptom on inoculation of *Okra yellow vein Bhubaneshwar virus* (OYBHUV) isolate. In addition, the results of this study are in agreement with the results obtained for other begomoviruses like *Cotton leaf curl virus* (CLCV) and *Tomato leaf curl virus* from Bangalore in Southern India (Nateshan *et al.*, 1996; Muniyappa *et al.*, 2000).

In the study, on release of viruliferous whiteflies at the rate of 20 whiteflies per seedlings, cent per cent insect transmission was obtained for both BYVMV and ToLCV. The conditions provided were the same for all the viruses with 24 h of AAP and IAP each and one-hour latent period in between AAP and IAP. Latent period is required for begomoviruses since whiteflies transmit them in a circulative persistent manner (Fiallo-Olive *et al.*, 2016). The results obtained in this study were in agreement with the findings of Mehta *et al.* (1994), Rajasri *et al.* (2011) and Muniyappa *et al.* (2003) where cent per cent transmission of the begomoviruses by *B. tabaci* could be attained with 24 h AAP and IAP. Although, Muniyappa *et al.* (2003) reported that AAP and IAP of 30 min and 10 min respectively were sufficient for successful transmission of PYVMV in contrast to results of Jayashree *et al.* (1999), who reported that whiteflies required only 5 min to acquire PYVMV. In general, all the studies confirmed that virus transmission frequency increased with longer AAP and IAP up to a maximum at 6 h. We had given AAP and IAP of more than 6 h in this study so as to obtain efficient transmission of the viruses. According to Muniyappa *et al.* (2000), the plausible reason for varied latent/incubation period was due to the seasonal/environmental factors.

Muniyappa *et al.* (2003) in their study observed that five viruliferous whiteflies per plant were sufficient to cause cent per cent transmission. Whereas, Jayashree *et al.* (1999) and Rajasri *et al.* (2011) found that 15 whiteflies were required to achieve cent per cent transmission which further confirmed the observations of this study.

Unlike BYVMV and ToLCV, only 60 per cent transmission was attained in case of PYVMV. The possible reason could be that in case of PYVMV transmission, the vector might not have acquired enough viral inoculum from the source plant sample in order to produce cent per cent transmission or the chance of escape of seedlings from whitefly feeding as 20 whiteflies were released on 4 seedlings in one

cage. The results obtained through the biological experiments confirmed that the viruses causing the diseases were WTG (whitefly transmitted Geminivirus).

### 5.3.2 Graft transmission

Graft transmission by wedge grafting method resulted in cent per cent transmission of the viruses and the symptoms appeared one-week after grafting. Hence it was confirmed that all the three viruses were graft transmitted. Reddy and Yaraguntaiah (1979) and Rajasri *et al.* (2011) had reported graft and dodder transmission of ToLCV. Moreover, the observations of the present study were in agreement with that of Singh *et al.* (2016) wherein okra seedlings after 9-10 days of inoculation with BYVMV expressed the symptoms and also resulted in cent per cent transmission through grafting. Ghevariya and Mahatma (2017) had reported successful transmission of BYVMV through veneer grafting.

### 5.3.3 Seed transmission

Compared to the other transmission studies, seed transmission was of upmost importance due to the fact that the sole propagation of all the three crops was done through seeds. Hence, seed transmission of these viruses was investigated. It was observed that none of the seedlings raised from seeds of infected plants could produce any kind of symptom, thus ruling out seed transmission of the viruses. Moreover, none of the studies conducted on the viruses reported seed transmission. But seed transmission was reported in other begomoviruses such as *Tomato yellow leaf curl virus* (TYLCV) by with 73 to 91 per cent transmission Eui-Joon *et al.* (2016) and *Bittergourd yellow mosaic virus* (BgYMV) with 78 per cent transmission by Manivannan *et al.* (2019). Hence, we could confirm that the viruses were spread from plant to plant by *B. tabaci* biotype B.

## 5.4 CROSS-INFECTION STUDY

In the current agricultural scenario, wherein multiple cropping system was preferred over monocropping, cross-infection of viruses became a matter of concern. As per the study, none of the seedlings inoculated by the viruses could produce any symptoms confirming that the viruses *viz.,* BYVMV, ToLCV and PYVMV did not cause cross-infection in okra, tomato and pumpkin seedlings. The observations of Muniyappa *et al.* (2003) and Singh *et al.* (2008) were on par with the observations of this study. According to them, insect transmission of PYVMV in tomato and okra seedlings did not produce any symptoms. They suggested that PYVMV had limited host range compared to other begomoviruses in India wherein the host range was limited to a few crops of *Cucurbitaceae* family and *Nicotiana* sp. However, Prakasha (2010) reported cross-infection of BYVMV in pumpkin seedlings inoculated with the virus using whiteflies. The seedlings were reported to express symptoms of vein clearing and netting after 15 to 20 days of inoculation.

## 5.5 IMMUNODETECTION

The serology-based detection became an integral part of large-scale agricultural surveys and disease eradication programs in the past, and it was still a popular approach for the detection of many viruses in important horticultural crops. Among the different serological detection techniques, DAS-ELISA (Polyclonal Antibodies (PAb) are used) is virus strain specific and necessitates the conjugation of every detecting antibody to an enzyme while TAS-ELISA is more efficient in detection of antigen than DAS-ELISA as it is more specific (Monoclonal Antibodies (MAb) are used).


### 5.5.1 Immunodetection by Enzyme linked immunosorbent assay (ELISA) method

Here for the serological detection of BYVMV, ToLCV and PYVMV both Double antibody sandwich (DAS)-ELISA and Triple antibody sandwich (TAS)-ELISA methods were used. DAS-ELISA was done using antibody specific to ToLCNDV while TAS-ELISA was done using antibody specific to *African cassava mosaic virus* (ACMV) and Sri *Lankan cassava mosaic virus* (SLCMV).

In this study, the presence of begomovirus was detected from all the infected samples confirming BYVMD, ToLCD and PYVMD as begomoviruses. The PYVMV infected samples were found to be more serologically related to ToLCNDV and ACMV since they produced positive reaction to these antisera. The result was on par with the observations of Muniyappa *et al.* (1991) wherein, PYVMV was detected by TAS-ELISA with ACMV. In case of BYVMV, both ACMV and SLCMV were found to be more serologically related to the virus compared to ToLCNDV since the reaction to the former gave higher OD value. Observations recorded by Naveen (2016) and Sohrab (2017) supported the above findings. Though all the tomato samples were found to be serologically related to ToLCNDV compared to ACMV and SLCMV, which might be due to the polyclonal nature of the antisera used. In case of serological detection on seeds none of the tested samples expressed positive reaction to any of the antisera used confirming lack of seed transmission of the virus.

The positive reaction of the viruses to the antisera might be due to the hypothesis that viruses in the same geographical region (e.g. India) have more similar epitope profiles than viruses from different geographical regions (McGrath and Harrison, 1995). However, PYVMV has a distinct epitope profile which differs in detail from the profiles of other viruses originating from the Indian subcontinent (Nateshan *et al.,* 1996; Harrison *et al.,* 1997; Swanson *et al.,* 1998).

### 5.5.2 Immunodetection by Dot immunobinding assay (DIBA)

DIBA was another serological method which was efficient in detection of the viruses similar to ELISA. In this study, all the representative samples of BYVMV, ToLCV and PYVMV produced positive reaction to antisera of ToLCNDV though at varying degrees depending upon their serological relation with the ToLCNDV as mentioned in the above section. Similar observation was recorded by Abou-Jawdah (2008) wherein detection of ToLCV was done using DIBA on the infected samples using antisera of ACMV, a begomovirus infecting cassava. Likewise, Pun *et al.* (1999) successfully employed DAC-ELISA on nitrocellulose membrane to detect BYVMV in infected okra plants.

## 5.6 MOLECULAR DIAGNOSIS AND CHARACTERIZATION

Reaction of the viruses with monoclonal and polyclonal antibodies could distinguish them only to a limited extent. The viruses could only be well distinguished by polymerase chain reaction (PCR) and nucleotide sequencing as suggested by Varma and Malathi (2003).

### 5.6.1. Isolation of genomic DNA

The whole genome extractions of BYVMV, ToLCV and PYVMV were done using modified CTAB method (Lodhi *et al.*, 1994).Though the DNA extractions of similar viruses were done differently by different scientists, in general, all extractions were done by CTAB method. For example, Rishishwar *et al.* (2015) performed DNA extraction of BYVMV by employing CTAB-Mucilage-free method put forth by Ghosh *et al.* (2009) while Venkataravanappa *et al.* (2015) did DNA extraction using CTAB method developed by Doyle and Doyle (1990).

The quality of DNA of the isolated viruses were analysed using biospectrophotometer. The study revealed that the quality of DNA was low for all the samples since the ratio of 260 nm to 280 nm readings noted ranged from 1.11

(ToLCV Vellayani) to 1.51 (ToLCV Mukhathala). Since all the readings were less than the desired value of 1.8 required for a good quality DNA, we can infer than there was high contamination of proteins in the DNA extracts. The quantification of the extracted DNA further revealed that it was not necessary that good quality DNA extract should have high concentration of DNA. For example, ToLCV Vellayani isolate with spectrophotometer reading 1.11 had 1138.5 ng $\mu l^{-1}$ of DNA while ToLCV Mukhathala isolate with spectrophotometer reading 1.50 had only 369.6 ng $\mu l^{-1}$ of DNA.

### 5.6.2 Amplification of coat protein gene of BYVMV, ToLCV and PYVMV

#### 5.6.2.1 Polymerase chain reaction (PCR)

Molecular detection of the begomovirus in the infected plant samples were done by PCR. For this, two primers specific to coat protein region of begomoviruses were used *viz.*, DENG forward and reverse primer (Deng *et al.*, 1994) and AV/AC primer (Wyatt and Brown, 1996) which amplified products of size 520 bp and 575 bp respectively.

#### 5.6.2.2 Gel electrophoresis

The PCR products were electrophoresed in 1.2 per cent agarose gel and analysed in gel documentation system.The molecular detection of seed samples collected from infected plants revealed that the viruses were present in tender seeds while they were not found in mature seeds. The viruses were amplified from both the seed coat and cotyledon samples of the three viruses while none of the above parts from mature seeds could yield amplicons of required size. It might be due to an increase in production of some virus inhibitory substance in mature seeds. Further study needs to be done for better understanding of the reason behind the contradicting results, though; molecular detection of the begomoviruses under study from seeds was not investigated. There were a few begomovirus which were detected in seed samples through molecular detection, one such example was *Dolichos yellow mosaic*

*virus* (DoYMV) (Suruthi *et al.*, 2018). DoYMV-CP specific primer could detect the virus in different parts of the seeds *viz.*, seed coat, endosperm and embryo. Similarly, TYLCV and BgYMV could also be detected from the seeds ( Eui-Joon *et al.*, 2016 and Manivannan *et al.*, 2019).

All the tested leaf samples could amplify amplicons of required size with both the primers and confirming that it was begomovirus causing BYVMD, ToLCD and PYVMD in Kerala. The results of this study were in general agreement with that observed by Singh *et al.* (2008) and Naveen (2016). They could also amplify a product size of 500 – 600 bp corresponding to the common region and part of coat protein from DNA-A of PYVMV and BYVMV respectively when PCR was performed using degenerate primers (DENG-F and R). The PCR products of all the samples amplified by AV/AC primer were further sequenced for characterization of the viruses involved with the diseases.

**5.6.3 Characterization of coat protein gene**

The BLASTn analysis of the coat protein sequences of the samples with the sequences in NCBI database revealed that the BYVMV isolates shared maximum sequence identity (99.42 %) with *Okra enation leaf curl virus*. A similar observation was recorded by Naveen (2016). Similarly, the CP gene sequence analysis of ToLCV isolates revealed that Vellayani and Neyyattinkara isolates expressed higher similarity to ToLCNDV (96.89 %) while the others to *Tomato leaf curl Kerala virus* (ToLCKeV) (99.05 %). But the PYVMV isolates showed higher sequence similarity to *Squash leaf curl China virus* isolate (98.63 %) suggesting that it might be SLCCNV associated with PYVMD in Kerala which agrees with the observations made by Singh *et al.* (2009) and Hamsa *et al.* (2016) though, Muniyappa *et al.* (2003) reported that PYVM disease in Karnataka state is caused by a strain of *Tomato leaf curl New Delhi virus*. The GenBank database accession numbers and details of the begomovirus showing higher nucleotide sequence similarity were mentioned in Table

24-26. BLASTx analysis for identification of the part of genome sequenced further confirmed that the amplified region was in fact CP gene of the corresponding viruses (Table 27-29).

### 5.6.4 Full genome amplification of DNA-A of BYVMV, ToLCV and PYVMV

#### 5.6.4.1 Rolling circle amplification (RCA)

RCA was a major breakthrough which revolutionized the discovery and characterization of single stranded circular DNA viruses especially begomoviruses (Haible *et al.*, 2006). It was used for the first time in cloning of a single-stranded circular DNA genome segment of a Begomovirus (Inoue-Nagata *et al.*, 2004). RCA utilizes the DNA polymerase of the *Bacillus subtilis* bacteriophage U29 (Blanco *et al.*, 1989; Dean *et al.*, 2001), which possesses polymerase and strand-displacement activity, allowing circular templates to be amplified preferentially (Lizardi *et al.*, 1998; Zhang *et al.*, 1998, 2001).

In this study the full genome of all the three viruses *viz.* BYVMV, ToLCV and PYVMV were amplified by RCA. It might be due to its simplicity, high sensitivity, and proofreading activity, the procedure was exploited to amplify small circular DNA sequences without initial knowledge of putative viral sequences present in the samples (Haible *et al.*, 2006; Lizardi *et al.*, 1998). For more than a decade the scientists continued to use RCA as a major detection technique for begomoviruses (Kumar *et al.*, 2008; Rajasri *et al.*, 2011; Wyant *et al.*, 2011; Rishishwar *et al.*, 2015).

#### 5.6.4.2 Restriction with different endonucleases

Restriction fragment length analysis (RFLA) enables to differentiate the virus isolates even without cloning and sequencing. Here, in this study the RCA products were restricted with *Bam*HI, *Hind*III, *Kpn*I, *Sal*I and *Xba*I restriction endonucleases which were commonly seen as unique restriction sites in begomovirus genome which

gives either 2.7 kb (DNA-A/DNA-B) or 1.3 kb (DNA β) fragments. Though in this study characterization of DNA-B or DNA-β were not done. Hence, further study need to be done to identify and characterize the presence of DNA-B and betasatellite.

In this study, the analysis of gel image revealed that 2.7 kb full genome of BYVMV could be obtained using *Hind*III, while ToLCV with *Hind*III, *Kpn*I and *Sal*I and in case of PYVMV with both *Bam*HI and *Xba*I confirming that it was indeed begomoviruses causing BTYMD, ToLCD and PYVMD. A similar observation was recorded by the scientists, Kumar *et al.* (2008), Rajasri *et al.* (2011), Wyant *et al.* (2011) and Rishishwar *et al.* (2015) in different begomoviruses.

**Development of primers for full genome amplification**

Amplification of full genome of the viruses could also be obtained using primers designed from the already existing begomovirus sequences of similar viruses in NCBI database by multiple sequence alignment (MSA) method.

In the present investigation a total of 22 begomovirus sequences (Table 31) of BYVMV, ToLCV and PYVMV from NCBI site were done MSA (Fig. 2) and from the regions that showed maximum similarity three sets of overlapping degenerative primers were designed (BIN-1, BIN-2, BIN-3, BIN-4, BIN-5 and BIN-6). PCR amplification was carried out and all the primers yielded amplicons of size 1300, 1000 and 900 bp corresponding to the three primers designed. The results of this study were in agreement with the findings of Patel *et al.* (1993), Singh *et al.* (2008), Venkataravanappa *et al.* (2012, 2013) and Rishishwar *et al.*, 2015). Though in this study PCR was followed by direct sequencing of amplicons unlike in other studies wherein the amplicons were cloned and further sequenced.

### 5.6.5 Characterization of full genome of DNA-A

The BLASTn analysis of the DNA-A full genome of BYVMV, TolCV and PYVMV was done in NCBI database. The study revealed that the BYVMV isolate of Vellayani shared maximum sequence identity to *Bhendi yellow vein mosaic virus* isolate OY173 segment DNA-A, complete sequence (Acc. No. - JQ326268.1) with 95.04 per cent similarity confirming that it is BYVMV involved in BYVMD in Kerala and named the virus as BYVMV-Vellayani.

The BLAST analysis of ToLCV isolate of Vellayani was found to be more similar to *Tomato leaf curl New Delhi virus* isolate TC237 segment DNA-A, complete sequence (Acc. No.- KF551582.1) with 92.89 per cent similarity, hence named as ToLCNDV-Vellayani. A similar finding was recorded by Rajasri *et al.* (2011) wherein the Hyderabad isolate of ToLCV was *Tomato leaf curl New Delhi virus* (ToLCNDV) strain, thus confirming the involvement of bipartite begomovirus causing ToLCD in tomato in South India which was otherwise detected only from North India. The homology of the viruses to ToLCNDV strain indicates the spread of ToLCV strains from North India to South India (Rajasri *et al.,* 2011) while ToLCD in Uttarakhad was caused by Tomato leaf curl Palampur virus with 99% nucleotide sequence identity (Anandhan *et al.*, 2011) which confirms that the same disease was caused by a large number of begomoviruses.

The PYVMV isolate of Mukhathala was found to me more related to *Squash leaf curl China virus* - [Pumpkin:Coimbatore] segment DNA-A, complete sequence (Acc. No.- AY184487.3) with 98.09 per cent similarity and hence named as SLCCNV-Mukhathala. The results obtained in the study suggest that PYVMD in India was caused by SLCCNV strain and not PYVMV. The observation was in agreement with the findings of Muniyappa *et al.* (2003), Singh *et al.* (2008) and Hamsa *et al.* (2016). Though there were reports of ToLCNDV and *Tomato leaf curl Palampur virus* (Tiwari *et al.,* 2011) associated with the disease in India. The

complete sequence of DNA-A of begomovirus isolated from pumpkin was 2756 nt long.

It was known that RCA/RFLP can give false positive results because RCA amplifies every small circular DNA present in the sample, and therefore, it amplifies plant material like mitochondrial mini plasmids in some plants (Homs *et al.*, 2008). However, in this study, such false positive results were not observed. All samples with a positive RCA/RFL pattern were confirmed to be positive for the viral infection by sequencing.

## 5.7 GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS

### 5.7.1 Genetic diversity analysis of coat protein gene

Phylogenetic relationship among coat protein sequences of all the begomovirus isolates constructed using the neighbor-joining method in the MEGA 7.0.26 software program revealed that all the three viruses *viz.,* BYVMV, ToLCV and PYVMV isolates were clustered together and hence related to each other. Further the phylogenetic trees confirmed the nucleotide sequences of same species would cluster together compared to other species. Apart from that it was observed that virus isolates of same species from a geographical area were clustered together compared to distantly isolated ones confirming that virus isolates of same geographical areas are more related to each other and as distance increases, variability too increases among the isolates as reported by Venkataravanappa *et al.* (2012).

Moreover, the phylogenetic comparison grouped the sequence of Vellayani and Neyyattinkara isolate of ToLCV with a cluster comprising all the PYVMV isolates confirming that these two virus isolates were more related to each other. It can be due to the fact that the ToLCV isolates of Vellayani and Neyyattinkara were suspected to be ToLCNDV while that of PYVMV to be SLCCNV. There were reports that both the viruses were more related to each other (Muniyappa *et al.*, 2003)

with their bipartite nature as a contributing factor for this high similarity or coat protein may probably be the highly conserved protein as the blastp analysis of the sequence showed highest similarity to both SLCCV and ToLCNDV with the same score (Hamsa *et al.,* 2016)

### 5.7.1 Genetic diversity analysis of DNA-A of BYVMV, ToLCV and SLCCNV

Phylogenetic tree was constructed with neighbour-joining method with 500 bootstrap replications for each of the viruses *viz.,* BYVMV, ToLCV and SLCCNV with selected isolates from NCBI database. The sequences wereselected based on the BLAST n analysis of each virus isolates under the study (Altschul., 1990) followed by Sequence alignments were performed using the Clustal W program (Thompson *et al.*, 1994). It was observed that each of the viruses were clustered with similar virus isolates of the same geographical area confirming that the isolates of samegeographical area were more related to each other than the ones found distantly. The GenBank database accession numbers and details of the DNA-A sequences of Begomovirus species used for comparison are mentioned in Table 32-34.

A phylogenetic comparison grouped the sequence of PYVMV with a cluster comprising all the ToLCNDV isolates and was supported by high bootstrap confidence scores confirming these two viruses were more related to each other compared to BYVMV. Muniyappa *et al.* (2003) also observed high similarity among ToLCNDV and SLCCNV isolates in his studies and concluded that they are more related to each other compared to other begomoviruses.

## 5.8 DEVELOPING SPECIFIC PRIMERS FOR THE BEGOMOVIRUSES

The specific primer development for each virus was a pre-requirement in early detection of the virus concerned with the disease. For this purpose specific primers were developed for BYVMV, ToLCV and PYVMV after the analysis of their open reading frames (ORFs). The ORF were determined by using the ExPASy

Compute pI/ Mw tool (http://www.expasy.org/tools/dna.html) and further the ORF region with maximum variability was found out using bioinformatic tools and primers were designed from that particular ORF for each virus. A similar method of primer designing was followed by Sivalingam and Varma (2007) and Zhou *et al.* (2008); and was successful in accomplishing virus detection of a particular species.

From this study we could conclude that in order to identify a virus, it is necessary to characterize the whole genome rather that its partial genome. For example the CP gene nucleotide analysis revealed that it might be Okra enation leaf curl virus causing BYVMD in Kerala while the whole genome characterization confirmed that it was BYVMV which cause the disease. Hence, a more understanding of the genome of the virus is necessary for its identification and at the same time for developing effective management strategies against the viruses like coat protein and movement protein mediated resistance.

Apart from this, we could also find that characterization of viruses based on the geographical area is necessary as variability among the virus isolates greatly depend on its geographical distribution. As there was high genetic variability among viruses causing the same disease from different locations like BYVMD was caused earlier by monopartite BYVMV but now there are reports of bipartite BYVMV causing the same disease. In this study characterization of viruses based on DNA-A alone was done, so further studies on biparitite/monopartite nature of the viruses and involvement of satellite DNA need to be done for the better understanding of the begomoviruses.

# *Summary*

# 6. SUMMARY

The present research work entitled 'Diversity of begomoviruses infecting major vegetable crops" was carried out in the College of Agriculture, Vellayani during 2017-2019, with the objective to identify the genetic variability in begomovirus isolates infecting okra, tomato and pumpkin and their different modes of transmission.

The infected samples were collected from different locations of Thiruvananthapuram and Kollam district and both disease incidence and vulnerability index were calculated from these locations. The disease incidence of bhendi yellow vein disease (BYVMD), tomato leaf curl disease (ToLCD) and pumpkin yellow vein mosaic disease (PYVMD) were observed to be highest (100 %) in most of the locations surveyed. The vulnerability index calculated using disease scoring of each disease revealed that the highest V.I. was recorded for BYVMD and ToLCD from Mukhathala (Kollam district) with values 86.67 and 85.00 respectively. While in case of PYVMD highest V.I. was observed in Tavanur of Malappuram (72.80).

In case of okra and pumpkin plants infected with yellow vein mosaic disease, vein clearing, severe mosaic along with general stunting and reduced fruiting were the common symptoms observed in all the surveyed locations. While in tomato plants infected with ToLCD, symptoms exhibited were leaf curling, stunting, distortion of leaves and reduced fruiting. Apart from these symptoms Neyyattinkara and Vellayani isolates of *Tomato leaf curl virus* (ToLCV) produced additional symptoms of yellowing, mottling and purplish discoloration of leaves.

The different modes of transmission of *Bhendi yellow vein mosaic virus* (BYVMV), ToLCV and *Pumpkin yellow vein mosaic virus* (PYVMV) on okra (Var. Varsha Uphar), tomato (Var. Vellayani Vijai) and pumpkin (Var. Ambili) seedlings revealed that all the three begomoviruses could be efficiently transmitted by whiteflies (100 %) provided with 24 h of acquisition access period (AAP) and inoculation access period (IAP). Graft transmission (100 %) of all the

viruses could be attained by wedge grafting. But seed transmission of the viruses were not observed as seeds collected from infected plants produced healthy seedlings without any symptom development even after one month. This, confirmed lack of seed transmission of the begomoviruses under the study.

The cross-infection study of the viruses using whiteflies revealed that none of the viruses were found to cause cross-infection in okra, tomato and pumpkin crops indicating lack of cross-infection nature of the viruses.

The serological and molecular detection were done on samples collected from the infected okra, tomato and pumpkin plants. Immunodetection by TAS-ELISA using antisera specific to *Tomato leaf curl New Delhi virus* (ToLCNDV) and DAS-ELISA using antisera of *African cassava mosaic virus* (ACMV) and *Sri Lankan casssava mosaic virus* (SLCMV) confirmed begomovirus infection in the samples, as we observed more than two fold increase in the absorbance value of disease sample compared to healthy. Similarly, immunodetection by DIBA using ToLCNDV antisera on representative begomovirus infected samples when analysed in GelDoc system further confirmed the virus infection as the intensity of colour development was higher for diseased samples compared to healthy sample. The begomoviruses were not detected in the seed samples using antisera, confirming lack of seed transmission of the viruses.

Molecular diagnosis of BYVMV, ToLCV and PYVMV were done by two sets of primers, specific to coat protein gene of Begomovirus *viz.,* AV/AC and DENG. Both the primers could amplify amplicons of 575 bp and 520 bp respectively in the leaf samples and tender seeds from infected plants confirming begomovirus infection. Neither of the primers could yield amplicons of required size in mature seed samples which further confirms lack of seed transmission of the viruses under the study.

Molecular characterization of Begomovirus isolates in the study using AV/AC primer revealed that begomovirus isolates from okra had more than 99.42 per cent similarity with other *Okra enation leaf curl virus* isolates in the NCBI database.

While molecular characterization of begomovirus isolates from tomato revealed that Vellayani and Neyyattinkara isolate of ToLCV had more similarity with ToLCNDV (96.89 %) while other isolates were found to share more similarity to *Tomato leaf curl Kerala virus* (ToLCKeV) (99.05 %) and *Tomato leaf curl Gujarat virus* (ToLCGuV) (98.11 %) isolates. The PYVMV isolates were more similar to *Squash leaf curl China virus* (SLCCNV) (98.63 %) isolates from the same geographical area.

Phylogenetic studies based on the CP gene sequence indicated a clear relationship between all the three Begomvirus isolates. The virus isolates from the same crop were clustered together indicating they were more related to each other with the exception of Vellayani and Neyyattinkara isolates of ToLCV; which were found to be clustered with PYVMV isolates in a different clade. It might to be due to fact that the ToLCV from Vellayani and Neyyattinkara shared highest similarity to ToLCNDV (bipartite virus) while the other isolates to ToLCKeV and ToLCGuV which were monopartite viruses. The genetic distance analysis revealed that maximum genetic variability was between Punalur isolate of BYVMV and Pappanchani isolate of PYVMV, while the least variablity (100 per cent similarity) was found between ToLCV isolates from Punalur and Tavanur.

The full genome (DNA-A) amplification of BYVMV Vellayani, ToLCV Vellayani and PYVMV Mukhathala were done using rolling circle amplification and intact ~2.7 kb full genomes of the viruses were obtained on restriction using different restriction enzymes. In order to characterize the full genome of DNA-A of the viruses, the viral DNA was amplified using three sets of overlapping degenerative primers designed by multiple sequence alignment of full genome of selected 22 begomovirus isolates from NCBI database. The three primer sets could amplify the required product size of 1000 bp, 1300 bp and 900 bp.

The characterization of full genome (DNA-A) revealed that the BYVMV isolate of Vellayani was more similar to BYVMV isolate (Acc. No. - JQ326268.1) with 95.04 per cent similarity confirming that it was BYVMV involved in BYVMD in Kerala and named the virus as BYVMV Vellayani. Similarly, ToLCV

isolate of Vellayani highest similarity to ToLCNDV isolate (Acc. No.- KF551582.1) with 92.89 per cent similarity, named as ToLCNDV Vellayani. The PYVMV isolate of Mukhathala was found to share highest nucleotide similarity to SLCCNV (Acc. No.- AY184487.3) with 98.09 per cent similarity and named as SLCCNV Mukhathala. From this we could infer that the virus associated with PYVMD was SLCCNV and not PYVMV.

The phylogenetic analysis of each of the viruses when compared with other similar virus isolates from NCBI database revealed that the BYVMV, ToLCNDV and SLCCNV isolates of Kerala were clustered together with similar virus isolate from same geographical area (i.e., Kerala, Tamil Nadu, Karanataka and Andhra Pradesh isolates were more related to each other). But when the phylogenetic analysis of the three viruses when analysed with the selected virus isolates revealed that all the three viruses were closely related to one another. Though ToLCNDV was clustered together with SLCCNV in a clade and hence they were more related to one another compared to BYVMV, which was clustered separately. The genetic distance analysis also suggested that the more geographically farther virus isolates were placed in different clades indicating the higher the genetic variability among them and vice versa.

From this study we observed that to identify a particular virus it was necessary to have specific primers developed for each of the viruses under study. Hence primers were designed using Primer 3 software from the ORF region that expressed highest variability.

The present investigation thus clearly demonstrate that it is necessary to characterize the whole genome of a virus to identify the virus associated with a disease rather than CP gene characterization, as it is not sufficient in identifying the virus. Moreover, the knowledge in structure of the virus gene could aid in design of mutant genes that possess increased efficacy and breadth in crop protection. So, there is an immediate need to characterize such hitherto unknown viruses and to develop a clear picture of the geographical distribution of these viruses.

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# *Appendices*

## APPENDIX I

### Buffers for DAS-ELISA

1. **Phosphate buffer saline (PBS - pH 7.4)**

| | |
|---|---|
| Sodium chloride | - 8.0g |
| Potassium dihydrogen phosphate | - 0.2g |
| Disodium hydrogen phosphate | - 1.1g |
| Potassium chloride | - 0.2g |
| Sodium azide | - 0.2g |
| Water | - 1 L |

2. **Wash buffer (PBS-T)**

   Add 250µL of Tween 20 to 500ml of PBS

3. **Coating buffer (pH 9.6)**

| | |
|---|---|
| Sodium carbonate | - 1.59g |
| Sodium bicarbonate | - 2.93g |
| Sodium azide | - 0.2g |
| Water | - 1 L |

4. **Antibody diluents buffer (PBS-TPO)**

   Add 20g Polyvinyl pyrrolidone and 2g ovalbumin to 1L of PBS-T

5. **Enzyme conjugate diluents buffer**

   Same as PBS-TPO

6. **Substrate solution (pH 9.8)**

| | |
|---|---|
| Diethanolamine | - 97mL |
| Sodium azide | - 0.2g |
| Water | - 800 ml |

## Appendix – II
## Buffers for DIBA

1. **Stock solution buffer (Tris-buffer saline, TBS – pH-7.5)**

   0.02M Tris – 4.84 g

   0.5M NaCl - 58.48g

   Adjust the pH to 7.5 with 1N HCl and make up the volume to 2 litre. This is used as wash solution

2. **Antigen extraction buffer (TBS-SDM)**

   Add 11.25g Diethyl dithiocarbamate (DIECA) to 1 litre TBS

3. **Blocking solution (TBS-SDM)**

   Add 5.0 g spray dried milk (SDM) to 100 ml of TBS

4. **Antibody and enzyme-conjugate diluent/buffer**

   Add antibody of required diluents to TBS-SDM

5. **Substrate buffer (pH 9.5)**

   0.1M Tris - 12.11 g

   0.1M NaCl - 5.85 g

   5mM $MgCl_2.6H_2O$) - 1.01 g

   Adjust the pH to 9.5 with 1N HCl and make up to 1litre.

6. **Substrate solution**

   Solution A

   Nitro blue tetrazolium (NBT) - 75mg

   Dimethyl formamide (DMFA) - 1ml

   Solution B

   Bromo chloro indolyl phosphate (BCIP) - 50 mg

   DMFA - 1 ml

   Store solutions A and B refrigerated in amber colour bottles. Add 44µl of NBT and 35µl of BCIP to 10 ml substrate buffer.

7. **Fixing solution**

   10mM Tris - 1.21 g

   1mM EDTA - 0.29 g

Adjust the pH to 7.5 with 1N HCl and make up to 1litre. All buffers contain 0.02% sodium azide as a preservative

## APPENDIX III

### Buffers for PCR products and Gel electrophoresis

**50 X TAE buffer**

Tris Base - 242 g

Glacial Acetic acid - 57.1 ml

0.5 M EDTA (pH 8.0) - 100 ml

Make up the volume to 1L using distilled water

**10 X TE buffer**

0.1 M Tris-Cl

0.01 M EDTA

Prepare 800 ml of distilled water in a suitable container

Add 15.759 g of Tris-Cl (desired pH) to the solution

Add 2.92 g of EDTA (pH 8.0) to the solution

Make up the volume to 1L using distilled water

**1.2 % agarose is prepared for casting and examination of PCR product**

## APPENDIX IV

### Sequences of CP gene of BYVMV, ToLCV and PYVMV under study using AV/AC primer

**Forward sequences**:

**>BYVMV_Chirayinkeezhu**

TGCCTCAATTAGCCCGCCTTGATGTTCCTAGAGGATGTGAAGGCCCAT
GTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATGGGTA
AGGTTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGACCCATC
GAGTAGGGAAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGA
TATGGATGGATGAGAATATTAAGACTAAGAACCATACGAACACCGTTA
TGTTTTGGATCGTGAGAGACAGGCGTCCTACAGGCACCCCCTACGATT
TCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCTACTGT
AAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTTCAGGC
GACAGTTACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCCAATTAG
GAAATTCTATCGTGTTAACAATTACGTGGTGTATAATCACCAGGAAGC
TGGGAAGTATGAAAATCACACTGAGAATGCTTTGTTGTTGTATATGGC
GTGTACACATGCCTCAAATCCC

**>BYVMV_Nedumangad**

TGCATGAATAAAAACCAAGGGTAATATTCGCAGGGGATGTCAAGGCC
CATGAAAAAATGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG
GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGACC
CATCGAGTAGGGAAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCA
AGATATGGATGGATGAGAATATTAAGACTAAGAACCATACGAACACC
GTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACAGGCACCCCCTAC
GATTTCCAGCAAGTGTTTAATGTTTATGACAACGAGCCTTGTACGGCT
ACTGTAAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTT
CAGGCGACAGTTACAGGAGGACAGTATGCTTGAAAGGAACAAGTTCC
AATTAGGAAATTCTATCGTGTTAACAATTACGTGGTGTATAATCACCA
GGAAGCTGGGAAGTATGAAAATCACACTGAGAATGCTTTGTTGTTGTA
TATGGCGTGTACTCATGCCTCAAATCCATCCTGGGCTTCCTATACATAG
GCATCT

**>BYVMV_Kottarakkara**

TCTTCATAGAATGTAACTCGGCCAGTTCCAGGCGATGTGAAGGCCCAT
GTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATGGGTA
AGGGTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGACCCATC
GAGTAGGGAAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGA
TATGGATGGATGAGAATATTAAGACTAAGAACCATACGAACACCGTTA
TGTTTTGGATCGTGAGAGACAGGCGTCCTACAGGCACCCCCTACGATT

TCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCTACTGT
AAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTTCAGGC
GACAGTTACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCCAATTAG
GAAATTCTATCGTGTTAACAATTACGTGGTGTATAATCACCAGGAAGC
TGGGAAGTATGAAAATCACACTGAGAATGCTTTGTTGTTGTATATGGC
CTGTACACATGCCTCAAACCCA

**>BYVMV_Mukhathala**
TTGCCAATCCCAAAATCACCACGGCATGATAACAGGCGATGTGAAGGC
CCATGTAAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACAT
GGGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGAC
CCATCGAGTAGGGAAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGC
AAGATATGGATGGATGAGAATATTAAGACTAAGAACCATACGAACAC
CGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACAGGCACCCCCTA
CGATTTCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCT
ACTGTAAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTT
CAGGCGACAGTTACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCC
AATTAGGAAATTCTATCGTGTTAACAATTACGTGGTGTATAATCACCA
GGAAGCTGGGAAGTATGAAAATCACACTGAGAATGCTTTGTTGTTGTA
TATGGCCTGTACTCATGCTTCCAACCCATTT

**>BYVMV_Neyyattinkara**
TCCAGAACTCGAAAATATGCATCAAACAGGCGAGCCAAAGGCTTATGT
AAAAATGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATGGGTAA
GGTTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGACCCATCG
AGTAGGGAAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGAT
ATGGATGGATGAGAATATTAAGACTAAGAACCATACGAACACCGTTAT
GTTTTGGATCGTGAGAGACAGGCGTCCTACAGGCACCCCCTACGATTT
TCAGCAAGTGTTCAATGTTTATGACAACGAGCCGTCTACGGCTACTGT
AAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTTCAGGC
GACAGTTACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCCAATTAG
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TGGGAAGTATGAAAATCACACTGAGAATGCTTTGTTGTTGTATATGGC
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**>BYVMV_Punalur**
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GTAAAAGGGCAGTCCTTTTGAGGCGAAGAACCATATTGGTCACATGGG
TAACGTTATCTGGTCTATCTGATGTTACTATGGGTATGGGGCTGACCCA
TCGAGTAGGGAAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAA
GATATGGATGGATGAGAATATTAAGACTAAGAACCATACGAACACCG
TTATGTTTTGGATCGTGAGAGACAGGCGTCCTACAGGCACCCCCTACG
ATTTCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCTAC
TGTAAATAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTTGA
GGCGACAGTTACTT

**>BYVMV_Vellayani**

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GATTTCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCT
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TCAGGCGACAGTTACAGGAGGACAGTATGCTTGTAAGGAACAAGTTC
CAATTAGGAAATTCTATCGTGTTAACAATTACGTGGTGTATAATCACC
AGGAAGCTGGGAAGTATGAAAATCACACTGAGAATGCTTTGTTGTTG
TATATGGCCTGTACACATGTTTTCCAACCCCA

**>PYVMV_Mukhathala**

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**>PYVMV_Tavanur**

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**>PYVMV_Kasaragod**

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**>PYVMV_Pappanchani**
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**>ToLCV_Chirayinkeezhu**
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**>ToLCV_Kottarakkara**
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**>ToLCV_Mukhathala**
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**>ToLCV_Nedumangad**
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**>ToLCV_Punalur**
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GATTTTTTCGTGTTAATAATTACGTAGTTTTATAATCACCAGGAAGCTG

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**>ToLCV_Vellayani**

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GGCATGCGACCGTGACGGGAGGAACATATGCATCTAGGGAGCAAGC
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**>ToLCV_Neyyattinkara**

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**>ToLCV_Tavanur**

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GATTTTTTCGTGTTAATAATTACGTAGTTTTATAATCACCAGGAAGCTG
GGAAGTACGAAAATCATACTGAGAATGCTTTGTTGTTGTATATGGCAT
GTACACATGCTTCAAATAACTCCT

## APPENDIX V

### DNA-A full genome sequences of BYVMV Vellayani, ToLCNDV Vellayani and SLCCNV Mukhathala

**>BYVMV-VELLAYANI-TOTAL NUCLEOTIDE**

ACCGGAGGCCGCGCGATTTTTTTGTGGGCCCCCGATTTATGAGATTGCT
CCCTCAAAGCTAAATAACGCTCCCGCCCACTATAAGTACTTGCGCACT
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CTTCGACAGCCCGTATTCAACCCGTGGAGTTGCCCCCAGTTGCCGGCA
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ACTCCAATGGCATAATTGTAAATATACAACTTTAATTTGAAATTCTCAC
GAACAGGTTAAAGCGGCCAGCCGTCTAATATT

ORF1-114-480
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TCCGGGTTCTGGTGGGCAGATCTTATTCGCGATCTTATTTTCTATTTTA
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CAGAAGTCCGGATGTTCCAAGGGGATG

ORF2-274-1045
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VGLTHRIGKRFCVKSVYVLGKIWMDENIKTKNHTNSVMFFLVRDRRPTD
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QALVKKFVRVNNYVVYNQQEAGKYENHTENALMLYMACTHASNPVYA
TLKIRIYFYDSVTN

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ORF3-COMPLEMENT 632-983
MGTSHIKHQRILGMILVFSCLLLVVNNIIVNPNKLLNQRLFLTRILSTSNGC
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GGTTAAGAAGTTTGTTAGGGTTAACAATTATGTTGTTTACAACCAGCA
GGAGGCAGGAAAATACGAGAATCATACCGAGAATGCGTTGATGCTTT
ATATGGCTTGTACCCAT

ORF4-COMPLEMENT 1049-1457
MDSRTGVRITAAQAKNGVYIWKVANSLYFKILNHDNRPFTTNMDIITLRI
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ORF5-COMPLEMENT 1154-1601

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GGTTGTCGTGGGCTGAGGATCTTGAAATAAAGGGAATTTGCAACCTTC
CAGATATAGACGCCATTCTTTGCTTGAGCTGCAGTGATGCGTACCCCT
GTGCGAGAATCCAT

ORF6-COMPLEMENT 1499-2453
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VSENIMDAAARPLRPWSLVLEGDSRTGKTMWARSLGPHNYLCGHLDLSP
KVYSNDAWFNIIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPVQIKGGI
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AQGSEETHQEEESRS

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ORF-7- COMPLEMENT 2132-2432
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GGCACTTGTATTTGCCCTCGAACTGGATGAGCACGTGAAGATGAGGTT
CCCCAT

**>ToLCNDV-VELLAYANI-TOTAL NUCLEOTIDE**

ACCGAATGGCCGCGGTGCCTTTTGTGTGGGTCCAGAACCAATGAAATT
CGAGATACATGGCTTATTTAATGCGTGGGGACCAATAAATAGACTTGC
TCACCAAGTTTGGATCCACAAACATGTGGGATCCATTGTTGCACGAAT
TTCCAGAAAGCGTTCATGGTCTAAGGTGCATGCTAGCTGTAAAATATC
TCCAAGAGATAGAAAAGAACTACTCTCCGGACACAATCGGATACGAT
CTTGTTCGTGACCTAATCTCTGTCGTCCGTGCCAGAAACTATGGTGAAG
CGTCCAGCAGATATCTACATTTCAACGCCCGCATCGAAAGCACGCCGA
CGACTCAACTTCGACAGCCCCTATGGAACTCGTGCAGTTGTCCCCATT
GCCCGCGTCACAAAGTCAAGAGCATGGGCGAACAGGCCCATGAACAG
AAAGCCCAGGATGTACAGAATGTACAGAAGCCCTGATGTCCCAAAAG
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TGGGCTAACTCATAGGGTTGGTAAGCGTTTCTGTGTTAAATCTGTTTAC
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ATCCGTGATCATGGCAGTTGATCGCAATGAAGTATGAACACCCGCACG
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CACCTTTGGGCTGAGATCTAGATGTCCACACAAGTAATTGTGTGGTCC
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TAAGAGAGCACTTTGGATAAGTGAGGAAGTAATTTTTGGCATATATTT
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ORF 1
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AHEQKAQDVQNVQKP

ORF 2
MVKRPADIYISTPASKARRRLNFDSPYGTRAVVPIARVTKSRAWANRPMN
RKPRMYRMYRSPDVPKGCEGPCKVQSFEQRHDISHIGKVMCISDVTRGIG
LTHRVGKRFCVKSVYVLGKIWRDENIKTKNHSNSVMFFLVRDRRHTGSP
QDFGEVFNMFDYEPSTATVKTMHRDRYQVFKKWHSTVTGGTYASKEQA
LVRKFVRVNNYVVYNQQEAGKYENHTENALMLYMACTHASNPVYATL
KIRIYFYDSVTN

ORF 3 -COMPLEMENT
MHGLHRCCARLIVKHVKNFPKILGGSCMTTVTDQKKHNTVRMVFSLDVF
IPPYLTQNVNRFNTETLTNPMS
ESSSSCNVTDTHDLANMRYVVPRFKRLHLTRAFTAPWHVGTSIHSVHSGF
SVHRPVGPGLCFCDAGNGDNCTSSIGAVEVESSAYLRCGR

ORF 4-COMPLEMENT
MITDSRTGEYITADRAESGVFIWEVPNPLYFKILKHDERPCLTNHDIITVQI
QFNHNLRKALGLHKCFLTFQIWTGLRPQTGRFLRVFKTQVLKYLDMLGV
VGINNVIRAAYHVLDNVLQRTVDVWATYDVKLNTY

ORF 5- COMPLEMENT
MQSSLHSRGHSIPVAKTSLPKKKKKSIRRRRVDLQCGCSYYISINCHDHGF
THRGIHHCGSSREWRVYLGSSKSPVFQNFETRRETMPNEPRHYNSPNTIQP
QPEESTGASQVFSNIPDLDGLTPSDWAFLESFQNPGP

ORF 6 -COMPLEMENT
MAPPKKFQIYAKNYFLTYPKCSLTKEEALSQLQTLETPTSKKFIKICRELHE
DGTPHIHVLIQFEGKFQCKNNRFFDLVSPSRSAHFHPNIQGAKSASDVKAY
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LAPKDYVLQFHNLITNLDRIFQPRSEVYVSPFSLSSFDRVPSELVDWVTSN
VLCAAARPFRPISVVIEGDSRTGKTMWARCLGPHNYLCGHLDLSPKVYSN
DAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPMMIKGGIPTIFL
CNKGPQSSYKGFLDEEKNAALKQWALKNAVFITLEGPLYSGRENVAPQE
EQEGDQTSTC

ORF 7-COMPLEMENT
MGLRISMFSSNLKENSSAKITDSSTWCPQVGQHISIRTFRELNQHRTSKHTS
IKKETF

**>SLCCNV-MUKHATHALA-TOTAL NUCLEOTIDE**

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ORF1 – 120-459
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ADEQKTQYGQNV

ORF 2- 280-1050
MAKRPADIIISTPASKVRRRLNFDSPYGARAVVPIARVTKAKAWTNRPMN
RKPSMDRMYRSPDVPRGCEGPCKVQFFESRHDVSHIGKVMCVSDVTRGT
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ALVRKFVRVNNYVVYNQQEPGKYENHTENALMLYMACTHASNPVYATL
KIRIYFYDSVTN

ORF 3- COMPLEMENT 1048-1458
MITDSRTGEYITADRAEDGVYIWEVPNPLYFKITGHDIRPCLEEHDIIKVQI
MFNHNLRKALGLHKCVLMFQIWTGLRPPIGIFLRVFKTQVLKYLDMLGVI
GIYNVLRAVYHVLDNVLEKTIDVGTTYDVKLNLY

ORF 4- COMPLEMENT 1193-1593
MQSSSQSKNHSIPVAKESLHPRKKRNIRRRRIDLPCGCSYYMSIDCHDHGF
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QPQPPESTGASQMCSNVPNLDGLTPSDWDFLESL

ORF 5- COMPLEMENT 1500-2585
MAPPRHFKKSAKPIFLTYPKCSITKEEALSQLRTLETPTSKKFIKICRELHED
GSPHIHVLIQFEGKFQCKNNRFFDLVSPSGSAHFHPNIQGAKSASDVKKYI
DKDGDVLEWGVFQIDGRSARGGQQTANDAYARAINTGNKEDALKVLKE
LAPKDYVLQFHNMNSNLDRIFPPRAEVYISPFSVSSFDSFPPELVDWVSGN
VVCSAARPIRPIGIVLEGDSRTGKTAWARSLGGHNYLCGHLDLSPRVYNN
DAWYNVIDDVDPHYLKHFKEFMGAQRDWQSNTKYGKPVMIKGGIPTIFL

RNKGPNSSYKEYLDEEKNAALKQWALKNAIFITIEEPLYSGRERIASSEEEE
EHQTQTN

ORF 6- COMPLEMENT 2252-2428
MGLRISTFLSSSKESSSARIIDSSTWYPQVGQHISIRTFRELNRHLTSRNTST
KTVTC

# Abstract

# DIVERSITY OF BEGOMOVIRUSES INFECTING MAJOR VEGETABLE CROPS

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**(2017-11-034)**

**Abstract of the thesis**
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**DEPARTMENT OF PLANT PATHOLOGY**

**COLLEGE OF AGRICULTURE**

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**MASTER'S DEFENSE SEMINAR**

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**ABSTRACT**

## Diversity of begomoviruses infecting major vegetable crops

The present study entitled "Diversity of begomoviruses infecting major vegetable crops" was carried out at College of Agriculture, Vellayani during 2017-2019 with the objective of immunomolecular detection and genetic analysis of the begomoviruses *viz.*, *Bhendi yellow vein mosaic virus* (BYVMV), *Tomato leaf curl virus* (ToLCV) and *Pumpkin yellow vein mosaic virus* (PYVMV). Plant samples showing the typical symptoms of bhendi yellow vein mosaic disease (BYVMD), tomato leaf curl disease (ToLCD) and pumpkin yellow vein mosaic disease (PYVMD) were collected from three taluks each of Thiruvananthapuram (Neyyattinkara, Nedumangad and Chirayinkeezhu) and Kollam (Kollam, Kottarakkara and Punalur) districts of Kerala during March-November, 2018. High disease incidence (DI) was observed in all the surveyed locations. High vulnerability index (V.I.) was recorded in BYVMD (86.6) and ToLCD (85) in Mukhathala of Kollam. Since PYVMD was observed only at Pappanchani of Thiruvananthapuram and Mukhathala of Kollam, the survey was extended to Tavanur of Malappuram and Pilicode of Kasaragod districts. High V.I. of PYVMD (72.8) was recorded in Tavanur.

Symptomatology of begomovirus infected crops revealed typical symptoms of BYVMD, ToLCD and PYVMD; like vein clearing, leaf curl and stunted growth. Vellayani and Neyyattinkara isolates of ToLCV produced additional symptoms *viz.*, purplish discoloration, mottling with yellowing and cupping of leaves in tomato.

All the three viruses were transmitted by whiteflies (*Bemisia tabaci* Gen.) and through grafting whereas none of the viruses were seed transmitted. Cross infection of these three viruses using whiteflies did not produce infection in any one of the crops studied.

Immunodetection of the three viruses was confirmed using polyclonal antisera of other begomoviruses *viz. Tomato leaf curl New Delhi virus* (ToLCNDV) and *Sri Lankan cassava mosaic virus* (SLCMV) through TAS (Triple antibody sandwich)-ELISA and DAS (Double antibody sandwich)-ELISA and dot immunobinding assay (DIBA) in infected leaf samples whereas the seeds from infected plants did not produce any positive reaction.

The quality and quantity of isolated DNA using Qiagen's DNeasy Plant DNA extraction Mini Kit was assessed using Bio-Spectrophotometer. The PCR based molecular



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detection using DENG and AV/AC primers specific to coat protein (CP) of begomovirus could yield amplicons of 520 bp and 575 bp respectively which confirmed the presence of the viruses in the infected leaf samples; whereas the seeds collected from infected plants did not yield any amplicons confirming the viruses were not seed transmitted.

The complete sequence of CP gene of the different isolates revealed maximum similarity of BYVMV isolates to *Okra enation leaf curl virus* (>99 %), ToLCV isolates to *Tomato leaf curl Kerala virus* (>99 %) and ToLCNDV (>96 %) but PYVMV isolates were more similar to *Squash leaf curl China virus* (SLCCNV) (>98 %). Further, phylogenetic analysis of the isolates of the three viruses revealed that they were closely related to each other as they were clustered together. But ToLCV isolates of Vellayani and Neyyattinkara were found to be closely related to isolates of PYVMV. Punalur and Tavanur isolates of ToLCV were more closely related to other BYVMV isolates. Genetic distance analysis among isolates of the viruses revealed that maximum variability was observed between Punalur isolate of BYVMV and Pappanchani isolate of PYVMV (0.528) while 100 per cent similarity was observed between ToLCV isolates of Punalur and Tavanur.

The full genome amplification and sequencing of Vellayani isolate BYVMV and ToLCV; and Mukhathala isolate of PYVMV were performed using three sets of overlapping degenerative primers. The blast analysis of full genome revealed that Vellayani isolate of BYVMV was closely related to other BYVMV (95.04 %); Vellayani isolate of ToLCV to ToLCNDV (92.89 %); and Mukhathala isolate of PYVMV to SLCCNV (98.09 %).

Phylogenetic analysis of the isolates of three viruses with other isolates of similar viruses from neighboring state and countries retrieved from NCBI database revealed that Vellayani isolates of BYVMV was closely related to other BYVMV isolates as they were grouped in a clad while Vellayani isolate of ToLCNDV was distantly related to other ToLCNDV isolates. ToLCNDV isolates were clustered in both BYVMV and SLCCNV suggesting that ToLCNDV may be the connecting link between BYVMV and SLCCNV which were grouped in different clades. The genetic distance analysis confirmed least divergence of evolution between ToLCNDV-Vellayani and SLCCNV-Mukhathala and greater divergence of evolution between BYVMV-Vellayani and SLCCNV-Mukhathala isolates. Among the three viruses, ToLCNDV is more related to SLCCNV compared to BYVMV. Therefore, phylogenetic analysis using whole genome is more reliable to decipher genetic relationship and diversity among viruses.

Specific primers for detection and identification of BYVMV, ToLCNDV and SLCCNV were developed through *in silico* analysis of whole genome sequences. The present study clearly demonstrates that PYVMD is caused by *Squash leaf curl China virus.*