CHARACTERISATION AND MANAGEMENT OF TOMATO LEAF CURL VIRUS IN KERALA

By

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(2012-11-121)

THESIS
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2014

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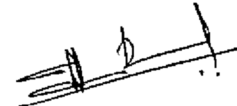
I hereby declare that the thesis entitled "**Characterisation and management of tomato leaf curl virus in Kerala**" is a bonafide record of research done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.



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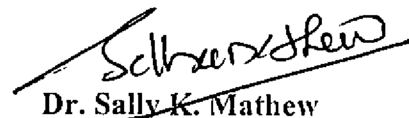

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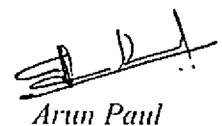

Arun Paul

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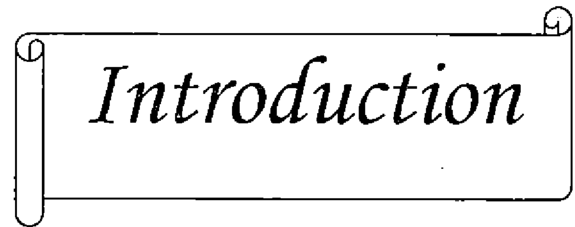
ABBREVIATIONS

A	- Adenine
ACMV	- *African cassava mosaic virus*
bp	- Base pair
BLAST	- Basic Local Alignment Search Tool
BGMV	- *Bean golden mosaic virus*
C	- Cytosine
cm	- Centimeter
CTAB	- Cetyl trimethyl ammonium bromide
CI	- Coefficient of Infection
CP	- Coat Protein
DAC-ELISA	- Direct Antigen Coating ELISA
DAP	- Days After Planting
DAS- ELISA	- Double Antibody Sandwich ELISA
DMRT	- Duncan's Multiple Range Test
DNA	- Deoxyribo Nucleic Acid
EDTA	- Ethylene Diamine Tetra Acetic Acid
ELISA	- Enzyme Linked Immuno Sorbent Assay
g	- Gram
G	- Guanine
h	- Hour
ICMV	- *Indian cassava mosaic virus*
KAU	- Kerala Agricultural University
kb	- Kilo base pairs
kDa	- Kilo Dalton
l	- Litre
m	- Metre
MAb	- Monoclonal Antibody
ml	- Millilitre
mM	- Millimolar
µg	- Microgram

µl	- Microlitre
min	- Minute(s)
M	- Molar
nm	- Nanometer
NCBI	- National Centre for Biotechnology Information
OD	- Optical Density
ORF	- Open Reading Frame
PBS	- Phosphate Buffered Saline
PBS	-T- PBS Tween
PCR	- Polymerase Chain Reaction
PDI	- Per cent Disease Incidence
PDS	- Per cent Disease Severity
PVP	- Poly Vinyl Pyrrolidone
RCA	- Rolling Circle Amplification
rpm	- Rotations per minute
sec	- Second(s)
SLCMV	- *Sri Lankan cassava mosaic virus*
T	- Thymine
TAE	- Tris Acctate EDTA
TAS-ELISA	- Triple Antibody Sandwich ELISA
TE	- Tris EDTA
ToLCD	- Tomato leaf curl disease
v/v	- Volume by volume
w/v	- Weight by volume


Introduction

1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most popular and widely grown vegetables in the world. Nutritionally, tomato is a significant dietary source of vitamin A and C. Furthermore, recent studies have shown the importance of lycopene, a major component of red tomatoes, which has strong antioxidant properties that can act against human diseases such as cancer and heart disorders.

Bacterial wilt, caused by *Ralstonia solanacearum,* is still considered as the major constraint for the cultivation of tomato in Kerala. Now, *Tomato leaf curl virus* (ToLCV) a heterogeneous complex of whitefly vectored geminivirus, has emerged as a serious production limiting factor in Kerala, results in severe crop loss during summer months. The impact of this disease is near total loss of the crop. Every year, ToLCV causes extensive damage to tomato crops all over the world worth millions of dollars. Sadasiva *et al.* (2006) estimated 70 to cent per cent yield loss due to this disease in India.

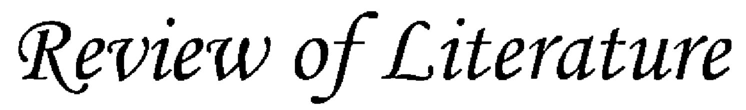
Geminiviruses form the second largest family of plant viruses and during the last two decades, these have emerged as the most devastating pathogen, of the tropics and subtropics threatening crop production and causing astronomic economic losses. Compared to other virus groups of the family *Geminiviridae*, begomoviruses have emerged as more serious problems in a variety of crops especially tomato. Major contributory factors for the emergence and spread of these viruses are the evolution of variants of the pathogen and increase in the vector population.

Considering the importance of tomato leaf curl disease in Kerala, Kerala Agricultural University has initiated research on developing resistant varieties against this disease. However, valid information on certain aspects like molecular variability and disease management strategies are lacking. In Kerala, various types of symptoms have been noticed on the leaf curl affected plants and such variation can be due to the

presence of different strains of the pathogen (Al-ani *et al.*, 2011). The knowledge on the existence of different strains is highly essential for disease resistance breeding.

Furthermore, great importance has to be given on developing effective management strategies for this devastating disease, which will be helpful to the farming community. Realising the importance of the above facts, the present study was undertaken giving emphasis on the following aspects.

- Occurrence of tomato leaf curl disease in major tomato growing tracts of Palakkad.
- Symptomatology.
- Serodiagnosis .
- Molecular characterisation.
- Disease management strategies.


Review of Literature

2. REVIEW OF LITERATURE

Tomato (*Solanum lycopersicum* L.) is one of the most popular and widely grown vegetables in the world ranking second in importance to potato in many countries. Pest and diseases are the major limiting factors in tomato cultivation. Tomato leaf curl virus disease has recently emerged as a major threat to the cultivation and production of tomato in Kerala.

2.1. Occurrence of *Tomato leaf curl virus* (ToLCV)

Tomato is affected by two types of leaf curl virus *viz. Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus* (ToLCV). Tomato yellow leaf curl disease was first reported from Israel by Avidov in 1940. Martinez *et al.* (2003) reported the infection of this disease around 7 million ha of crop plants in 40 countries and so far it is not reported from Indian subcontinent (Rishi, 2004; Glick *et al.*, 2009).

Globally, *Tomato leaf curl virus* has also been reported from Taiwan (Green *et al.*, 1987), Australia (Dry *et al.*, 1993), Bangladesh (Begum and Khan, 1996), Bhutan (Thinlay and Pinjoe, 1996), Sri Lanka (Zoysa, 1996) and Nepal (Joshi *et al.*, 1996).

In India, occurrence of leaf curl disease was first observed in the northern plains by Pal and Tandon (1937) and later reported by Pruthi and Samuel (1939). Vasudeva and Samraj (1948) also reported the incidence of ToLCV in northern India and later it was reported from Delhi (Vasudeva, 1959), Maharashtra (Varma, 1959), Coimabatore (Ramakrishnan *et al.*, 1964), Karnataka (Govinda, 1964), Kanpur (Singh and Lal, 1964), Kerala (Nair and Wilson, 1969), Punjab (Butter and Rataul,

1973), Lucknow (Srivastava *et al.*, 1975), Hissar (Varma and Poonam, 1977) and Pantnagar (Sakalani and Mathai, 1978).

2.2. Importance of ToLCV

Tomato leaf curl disease causes millions of dollars loss to tomato crop all over the world every year. In India also ToLCV was reported to be a serious disease on tomato. Sastry and Singh (1973) observed that, ToLCV affected plants produce very few fruits when infected within 20 days after transplanting and resulted upto 92.30 per cent yield loss. Whereas, plants infected at 35 and 50 days after transplanting showed 74 and 22.9 per cent yield loss respectively. Banerjee and Kalloo (1987) reported that, the major constraint for the cultivation of tomato was the outbreak of ToLCV during summer in south India and autumn in north India.

More than 90 per cent yield loss occurs when infection initiates within four weeks of transplanting (Saikia and Muniyappa, 1989). Rataul and Brar (1989) found that, the infection occurs at all stages of growth, causing 17.6- 99.7 per cent yield loss, depending on the stage of the crop at the time of infection. Gururaj and Madalageri (2002) observed 50-70 per cent and Sadasiva *et al.* (2006) noted 70-100 per cent yield loss due to ToLCV infection.

The report from Sudan revealed the severity and significance of this disease, where it resulted in 75 per cent or more reduction in yield and posed as a national problem (Yasin and Nour, 1965).

2.3. Symptomatology

Vasudeva and Samraj (1948) described the symptomatology of tomato leaf curl disease. The infected plants showed vein clearing, reduction in leaf size, stunted

growth, deformation of leaflets, inward and outward curling, puckering of leaflets. The diseased plant usually developed purple patches especially on older leaves. Similar symptoms were also observed by various other researchers (Singh and Lal, 1964; Sastry and Singh, 1973; Raychaudhari and Nariani, 1977; Sakalani and Mathai, 1977; Reddy, 1978; Muniyappa, 1980; Muniyappa and Veeresh, 1984; Saikia and Muniyappa, 1989). Nariani (1968) observed enation on the lower surface of the ToLCV infected leaves in addition to other symptoms. Reddy *et al.* (1981) observed five distinct symptoms like leaf curling, chlorosis, purpling and shortening of internodes.

Muniyappa (1994) codified symptoms into five categories and designated as group I, II, III, IV and V. Group-I included symptoms of pale yellow color, vein clearing; upward curling; reduced leaf size, bushy appearance due to shortening of internodes and more number of lateral branches. Group II comprised of symptoms like vein clearing, upward curling, twisting of petioles, narrowed leaves, stiff and erect lower leaflets, leaf rolling and purpling of veins, whereas group III consisted of symptoms similar to group-II except the twisting of the petioles, greening of the veins and vein lets and presence of enation on the under surface of the leaves. Group IV included downward curling and severe twisting of petioles, branches and flower sterility symptoms. In group V, plants appeared normal and only internal leaves showed slight curling and pale yellowing.

Nazeem *et al.* (2006) reported four different types of symptoms associated with ToLCV in Kerala. Reddy (2006) described the symptoms of ToLCV on Megha variety of tomato, as upward curling of the lower leaves, reduction in size of newly emerging leaves, slight chlorosis, crinkling and puckering. The plants developed slight purple pigmentation on curled leaf margin and plants exhibited partial or complete sterility.

Different kinds of disease symptoms like mild to severe downward and upward curling, blistering, crinkling and narrowing of leaves with stunted growth were observed on ToLCV infected plants in Assam and West Bengal by Saha *et al.* (2014).

2.4. Causal agent

Tomato leaf curl disease is caused by a whitefly transmitted geminivirus (WTG) commonly referred to as *Tomato leaf curl virus* (ToLCV). The virus is classified under the genus *Begomovirus* of *Geminiviridae* family.

Several species and strains of *Begomovirus* causing ToLCD have been reported (Fauquet *et al.*, 2003). Nearly 60 recognized begomoviruses and a large number of their strains, isolates and recombinants have been shown to cause diseases of tomato in Africa, Asia, Australia, North, Central and South America, and some parts of Europe (Fauquet *et al.*, 2008, Varma *et al.*, 2011). In Indian subcontinent, 12 species of the genus *Begomovirus* are known to be associated with ToLCD. They are *Tomato leaf curl New Delhi virus* (ToLCNDV) (Padidiam *et al.*, 1995), *Tomato leaf curl Bangalore virus* (ToLCBV) (Muniyappa *et al.*, 2000), *Tomato leaf curl Karnataka virus* (ToLCKV) (Chatchawankaphanich and Maxwell, 2002), *Tomato leaf curl Gujarat virus* (ToLCGV) (Chakraborty *et al.*, 2003), *Tomato leaf curl Bangladesh virus* (ToLCBDV), *Tomato leaf curl Sri Lanka virus* (ToLCSLV), (Rishi, 2004), *Tomato leaf curl Joydebpur virus* (ToLCJV) (Maruthi *et al.*, 2006), *Tomato leaf curl Palampur virus* (ToLCPMV) (Kumar *et al.*, 2008), *Tomato leaf curl Kerala virus* (ToLCKeV) (Pandey *et al.*, 2010), *Tomato leaf curl Patna virus* (ToLCPaV) (Kumari *et al.*, 2010), *Tomato leaf curl Pune virus* (ToLCPuV) and *Tomato leaf curl Rajasthan virus* (ToLCRV) (Varma *et al.*, 2011).

2.4.1. Genomic organization

The geminate nature of ToLCV was first reported from India by Muniyappa *et al.* (1991). Begomoviruses have circular single stranded DNA bipartite genome components, DNA-A and DNA-B, each with size of 2500-2800 nt. Only few begomoviruses have monopartite genome, which lack DNA-B. The existence of DNA β, a single-stranded circular satellite DNA was reported in some members of *Begomovirus* (Saunders *et al.*, 2000; Briddon *et al.*, 2003 and Zhou *et al.*, 2003). Recently, *Tomato leaf curl Patna virus* with β satellite affecting tomato was reported by Kumari *et al.* (2010). The most significant variation in the genomes of begomoviruses is the lack of DNA-B in monopartite begomoviruses.

Unlike the other white fly transmitted geminiviruses, *Tomato yellow leaf curl virus* (TYLCV) from Israel (Navot *et al.*, 1991), Spain (Noris *et al.*, 1994b), Sardinia (Kheyr- Pour *et al.*, 1991) and *Tomato leaf curl virus* (ToLCV) isolate from Australia (Dry *et al.*, 1993) have only DNA-A component. In contrast, a TYLCV-Thailand isolates (Rochester *et al.*, 1990) and ToLCNDV (Padidiam *et al*, 1995), ToLCGV (Chakraborty *et al.*, 2003) and ToLCPMV (Kumar *et al.*, 2008) from north India have been found to have both the DNA-A and DNA-B. Chakraborty *et al.* (2003) also cloned and sequenced DNA-A and DNA-B components of Varanasi and Mirzapur isolates and identified as ToLCGV. Whereas, the south Indian isolates were monopartite with only DNA-A (Muniyappa *et al.*, 2000; Pandey *et al.*, 2010).

In bipartite WTGs, both the genomic components are essential for infection. DNA-A codes for coat protein and replicase gene, whereas DNA-B codes for movement function (Townsend *et al.*, 1986: Davies and Stanley, 1989; Sung and Coutts, 1995; Hanley-Bowdoin *et al.*, 1999). DNA β is found to be associated with the severity of the symptom (Glick *et al.*, 2009; Sivalingam and Varma, 2012).

DNA-A contains four genes, AV1,AC1, AC2 and AC3 in which AV1 gene codes for virus coat protein (CP), AC1 codes for replication initiation protein (Rep)

which is essential for DNA replication, AC2 is a transcriptional activator for the virion-sense genes in both DNA-A and DNA-B components and AC3 has no role in infectivity but enhances the viral replication. The DNA-B has BV1 and BC1 which together code for virus movement (Rishi, 2004 and Glick *et al.*, 2009). The DNA β molecule is about 1.3 kb in length and expression of the βC1 protein resulted in considerable increase in symptom severity of the virus. This protein is suggested to act as a suppressor of gene silencing (Cui *et al.*, 2005).

2.5. Transmission of ToLCV

ToLCV is reported to be non sap transmissible (Vasudeva and Samraj, 1948). However, bipartite *Begomovirus* infecting tomato in north eastern Mexico was reported to be sap transmitted to tomato, *Nicotiana benthamiana* and common bean (Paplomatas *et al.*, 1994). Sohrab *et al.* (2013) also found that, tomato leaf curl New Delhi (ToLCNDV) inciting yellow mosaic disease in *Luffa* was sap transmissible and opined that sap transmission for most of the *Begomovirus* is difficult, except a few. ToLCNDV is a bipartite virus and due to the presence of the DNA B genes (involved in virus movement), such viruses tend not to be phloem limited and thus likely to be mechanically transmissible.

Graft transmission of ToLCV was reported by Vasudeva and Samraj in 1948 and many workers adopted it for screening (Divakaran, 2007 and Yadav, 2011). Reddy and Yaraguntaiah (1981) reported that, Dodder, *Cuscuta reflexa* could transmit ToLCV from one plant to another.

According to Makkouk (1978), Nakhla *et al.* (1978) and Nitzany (1975) *Tomato leaf curl virus* was not transmitted through seeds. The experiments conducted by Makkouk and Laterrot, (1983) also revealed that, ToLCV is not transmitted through soil.

ToLCV is transmitted in nature by whitefly, *Bemisia tabaci* (Vasudeva and Samraj, 1948; Varma, 1963; Cohen and Nitzany, 1966; Nariani, 1968; Makkouk *et al.*, 1979; Bharadwaj, 1992; Ramappa, 1993; Muniyappa, 1994; Caciagli *et al.*, 1995; Naik *et al.*, 2004; Rajasri *et al.*, 2011). The greenhouse whitefly, *Trialeurodes vaporariorum* and aphid, *Myzus persicae* can acquire the virus non-specifically but are not able to transmit it (Antignus *et al.*, 1994).

Cohen and Harpaz (1964) reported that, although ToLCV was transmitted in a persistent and circulative manner, it was not transmitted transovarially. However, the nymph of whiteflies could acquire the virus and transmit it upto the adult stage. This virus retention supports its circulative mode of transmission. The nymphs are as effective as the adults in acquiring the virus. Female transmission efficiency has been found to be five to six folds higher than that of males (Cohen and Nitzany, 1966: Ramappa, 1993; Caciagli *et al.*, 1995, Naik *et al.*, 2004). A single whitefly is able to transmit the virus (Mansour and Al-musa, 1992).

Minimum acquisition and inoculation feeding periods are 15-30 minutes, and the latent period in the vector is atleast 21 h. Once the whiteflies become viruliferous, they remained infective for 14 days (Butter, 1976) whereas, according to Reddy and Yaraguntaiah (1981) the virus is retained throughout the life span of the vector.

According to Naik *et al.* (2004) five adult whiteflies were required for cent per cent transmission of ToLCV from tomato to tomato, when acquisition and inoculation access periods were 24 h each. The minimum acquisition access and inoculation access periods were 10 and 20 min. respectively. A latent period of 6 h was necessary for *B. tabaci* to become viruliferous.

Rashid *et al.* (2008) noted that, when 3, 5 and 10 viruliferous whiteflies per plant were released, the disease transmission was 20, 30 and 70 per cent, respectively. Cent per cent transmission of TYLCV could be obtained with 15 whiteflies. Rajasri *et*

al. (2011) reported 20 per cent transmission of ToLCD with a single adult whitefly and cent per cent was obtained with 15 adults /plant.

2.6. Host range

Vasudeva and Samraj (1948) reported that, ToLCV exhibited leaf curl symptoms on *Nicotiana tobaccum* L. (cv. white barley, samsun and Harrison's species), *N. sylvestris* L. (cv. Spegas), *N. glutinosa* L., *Solanum tuberosum* L. (cv. Craig defiance) and *Datura stramonium* L. when inoculated by grafting.

Varma (1959) observed the transmission of ToLCV to *N. rustica* L., *Zinnia elegans* Jacq., *Datura stramonium* L., *Salvia spendons* selle, *Althaea rosea* Cav., *Petunia hyrbida*, *Euphorbia geniculata* L. and *Cassia tora* L. through whitefly.

Sastry (1984) noticed weed hosts such as *Acanthospermum hispidum, Ageratum conyzoides, Parthenium hysterophorus, Datura stramonium, E. geniculata* and *Gynandropsis pentaphylla* as sources of inoculum for tomato leaf curl disease.

Saikia and Muniyappa (1989) found *A. hispidum* DC., *A. conyzoides* L., *Bidens biternata, Chenopodium anthelmintium* (L.) Kuntze, *Conyza stricta, Garhinsoga parviflora* Cav., *Sonchus brachyotis* L., *Synedrella nodiflora* (L.) Gaertn., *Z. elegans* Jacq, *E. geniculata* L., *A. rosea* Cav., *Oxalis acetosella* L., *Capsicum annuum* L., *D. stramonium* L., *L. esculentum* L., *L. glandulosum* L., *L. hirsutum* L., *L. peruvianum* L., *N. benthamiana* L., *N. glutinosa* L., *N. tobaccum* L., *Physalis minima* L. and *S. nigrum* L. as collateral hosts of ToLCV.

Naik (2001) reported the natural occurance of ToLCV on several weeds *viz*., *A. hispidum* DC., *A. conyzoides* L., *O. acetosella* L., *O. corniculata* L., *E. geniculata* L., *S. hodiflora* (L.) Gaertn., *Galinsoga parviflora* Cav., *S. nigrum* L., *C. uniflora* L., *P. hysterophorus* L. and *Walteria americana* L. during all the seasons, but with high

disease incidence during summer as compared to kharif and rabi. High incidence ToLCV (37.68 per cent) was recorded on *E. geniculata* L., which is reported to be a reservoir host for ToLCV.

Khan *et al.* (2002) reported the occurrence of ToLCV on sunn hemp plants. Morilla *et al.* (2005) and Polston *et al.* (2006) observed the infection of TYLCV on chilli and demonstrated the ability of chilli plants to serve as symptomless reservoir. ToLCVs were reported on chilli, cowpea, okra and tobacco by Reddy *et al.* (2005) *Tomato leaf curl New Delhi virus* (ToLCNDV) isolate was found to infect bitter gourd (Tiwari, *et al.*, 2010), brinjal (Pratap *et al.*, 2011) and sponge gourd (Sohrab *et al.*, 2013).

2.7. Serodiagnosis

Since the development of the microplate method of Enzyme Linked Immunosorbant Assay (ELISA) (Clark and Adams, 1977), has become a routine method for detection and quantification of virus level in the plant (Stein *et al.*, 1983; Duan, 1995). ELISA is simple to perform, sensitive, and easily used for large scale detection. Several modifications and refinements have been done in ELISA techniques since its inception (Cooper *et al.*, 1986; Gow and Williams, 1989; Al-Bitar and Lusioni, 1995). They mostly differ in the quality and specificity of the reagents.

Sequeira and Harrison (1982) used ELISA technique for the detection of *Cassava latent virus* (CLV). They reported that, CLV was detected readily in the extracts of systemically infected *Nicotiana benthamiana* leaves but was less in extracts from leaves of naturally infected cassava. Cohen *et al.* (1983) found that, purified *Squash leaf curl virus* (SLCV) reacted with the SLCV antiserum and also with cassava latent virus in ELISA but not with the antisera of *Beet curly top virus*,

Horseradish curly top virus, Bean golden mosaic virus and *Tomato yellow dwarf virus.*

Monoclonal antibodies (MAbs) developed for *African cassava mosaic virus* (ACMV) have been used for the detection of three geminiviruses which occurred in Europe (Thomas *et al.*, 1986).

Mathew (1989) successfully used ELISA to detect *Indian cassava mosaic virus* (ICMV) in the crude sap of infected cassava, *N. benthamiana,* and *N. tobaccum* cv. Jayasri using ICMV-H antiserum.

Mandal (1989) succeeded in detecting *Croton yellow vein mosaic* (CYMV) using ACMV antiserum. Kounounguissa *et al.* (1989) observed the virus distribution in the cassava plant by ELISA, and the cassava leaves were found to have higher ELISA readings than the petioles or the stems. It was also noted that, younger leaves harvested from the top of the stems gave better results and amount of viral antigen decreased from the top to the bottom of the stem.

Muniyappa *et al.* (1991b) used different monoclonal antibodies of ACMV and ICMV to detect and characterize tomato leaf curl virus and opined that, the viruses could be well differentiated based on epitope profile using MAbs raised against ACMV and ICMV.

Swanson *et al.* (1992) reported 60 per cent conservation in the coat protein gene and hence polyclonal antibody raised against any *Begomovirus* could react with all begomoviruses. ISEM and ELISA detection of begomoviruses associated with yellow mosaic and yellow vein mosaic disease of many plants in India have been developed using polyclonal antibody to ICMV, ACMV and SqLCV.

Noris *et al.* (1994a) tested several methods, based on both antibodies and non-radioactive DNA probes and found that, indirect plate-trapping ELISA was only effective in detecting the TYLCV in purified preparations and not in crude extracts.

Swanson *et al.* (1998) raised a panel of 25 monoclonal antibodies (MAbs) against particles of two heterologous whitefly transmitted geminiviruses (*Begomovirus*) and used in triple antibody-sandwich ELISA (TAS-ELISA) to determine the detectability and epitope profiles of 26 Indian isolates of *Tobacco leaf curl virus* and 13 isolates of *Croton yellow vein mosaic virus*. They suggested that, TAS-ELISA with MAb SCR 18 was the most sensitive test for detecting Indian TLCV isolates than double antibody-sandwich ELISA with polyclonal antibodies.

Salim and Bandumala (2001) could detect a geminivirus by TAS ELISA from 13 samples of cassava, showing mosaic symptoms. The MAbs used in their study (SCR 60) was produced against an isolate of ICMV and opined that, as serological relationships were common among geminiviruses, the identity of virus could not be confirmed by polyclonal antibodies used in indirect ELISA tests.

Abouzid *et al.* (2002) produced polyclonal rabbit antisera for the coat protein of *Bean golden mosaic virus* Brazil isolate (BGMV), *Cabbage leaf curl virus* (CabLCV), *Tomato yellow leaf curl virus* (TYLCV), and *Tomato mottle virus* (ToMoV) and found that, polyclonal antisera prepared to express *Begomovirus* coat proteins were useful for the detection of begomoviruses in an array of assays.

Serological analysis of two types of tomato yellow leaf curl symptoms collected from different locations in Egypt was carried out using the whitefly transmitted geminivirus antiserum [immunogenic oligopeptides coat protein (Cp-3)] by El-Din *et al.* (2003). This experiment proved that, the putative TYMV belongs to whitefly transmitted geminivirus and both were different viruses having similarity in their antigenic properties of viral coat protein.

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was used by Farag *et al.* (2005) to confirm the identity of the virus isolated from leaf curl infected squash plants. Polyclonal antibodies specific to SLCV (geminivirus) reacted positively in DAS-ELISA and no serological reactions were

observed with other cucurbit viruses like *Zucchini yellow mosaic virus* (Potyvirus), *Squash mosaic virus* (Comovirus), *Cucumber mosaic virus* (Cucumovirus), *Watermelon mosaic virus* (Potyvirus), *Moroccan watermelon mosaic virus* (Potyvirus) and *Papaya ring spot virus* (Potyvirus).

Devaraja *et al.* (2003) successfully produced monoclonal antibodies to ToLCV and detected *Begomovirus* infection in tomato samples, and other crop species. Devaraja *et al.* (2005) standardized the detection of *Tomato leaf curl Bangalore virus* (ToLCBV) using DAS and TAS- ELISA techniques.

Studies conducted by Zacharia (2006) using DAC-ELISA showed that, antigen of *Bitter gourd distortion mosaic virus* (BGDMV) at 10^{-1} dilution reacted with monoclonal antibodies of *Squash leaf curl virus* (SLCV-0614/1) and *Tomato yellow leaf curl virus* (TYLCV-546/1) and established the serological relationship of BGDMV with these geminiviruses.

Reddy (2006) detected ToLCV in tomato samples collected from different parts of Karnataka by adopting DAC-ELISA technique and found that value of absorbance vary with sample to sample.

Njock and Ndip (2007) reported that, ACMV could not be detected by ELISA from the lignified tissues of cassava, but could be detected from young leaves and axillary branches.

Malekzadeh *et al.* (2011) tested tomato leaf samples collected from different places of Iran showing leaf curling, yellowing and stunting, for the presence of TYLCV using TAS- ELISA and found that, 39 per cent samples were infected with TYLCV.

Suresh *et al.* (2012) carried out ELISA with okra leaf samples, showing mosaic, necrosis and yellow vein mosaic symptoms, collected from commercial fields in Nasik district of Maharashtra state. The samples were tested for different viruses

and all fifteen samples showed positive reaction to antiserum specific to geminiviruses in DAS- ELISA.

Indra *et al.* (2013) adopted TAS- ELISA for the detection of tomato leaf curl disease using commercial polyclonal and monoclonal antibodies specific to ICMV and ACMV. ToLCV reacted with eight of the 14 MAbs specific for different epitopes in the particle protein of ACMV and two of the five MAbs specific to particle protein of ICMV.

2.8. Nucleic acid based detection

Viral nucleic acid-based techniques like dot-blot hybridization assays and polymerase chain reaction (PCR) are more sensitive than other methods. Availability of these diagnostic methods provides greater flexibility, increased sensitivity, and specificity for rapid diagnosis of plant viral diseases.

The affinity of one strand of DNA for its complementary sequence is one of the strongest and most exquisitely specific interactions found in nature. This specificity has been exploited in developing nucleic acid hybridization assays, which are based on the homology between two strands of nucleic acid. The dot- or spot-blot hybridization assay is a commonly used technique in plant virus diagnostics (Maule *et al.*, 1983; Garger *et al.*, 1983; Rosner *et al.*, 1986; Navot and Czosnek, 1989).

Tomato yellow leaf curl virus DNA was used as a probe to identify and analyse the virus related DNA in infected tomato plants and in the whitefly vector by squash blot technique (Czosnek *et al.*, 1988). Muniyappa *et al.* (2000) used radiolabeled ToLCV-Ban4 DNA as a probe in Southern blot hydridizations to detect viral DNA in plants. In addition to ToLCV, this probe detected other whitefly-trasmitted geminiviruses and suggested that, ToLCV could be detected in infected tomato plants and whiteflies by this hybridization method. Tripathi (2000) employed

nucleic acid spot hybridization (NASH) to study ToLCV isolates and agroinfections of ToLCV were detected in agroinoculated plants with different agroconstructs.

The sensitivity of nucleic acid-based detection systems was greatly improved following the development of the polymerase chain reaction (PCR) (Mullis *et al.*, 1986). PCR is an *in-vitro* method for amplifying target nucleic acid sequences. The speed, specificity, sensitivity, and versatility of PCR made it suitable in biological research. Since PCR can amplify the target nucleic acid present at an extremely low level and form a complex mixture of heterologous sequences, it has become an attractive technique for the diagnosis of plant virus diseases (Henson and French 1993; Candresse *et al.*, 1998).

Navot *et al.* (1992) amplified the genomic DNA molecule of an Israel isolate of TYLCV from DNA extracts of TYLCV infected *Lycopersicon esculentum* M-82 by polymerase chain reaction using synthetic oligonucleotides complimentary to different regions of the viral genome as primers. Detection of TYLCV in tomato tissues by a procedure based on PCR technology was reported by Martino *et al.* (1993). A pair of synthetic oligonucleotides derived from the sequence of TYLCV strain was used for the amplification of 750 bp band. Nakhla *et al.* (1993) employed PCR technique and characterized four tomato yellow leaf curl virus isolates of Egypt using specific primers of TYLCV-ICR (Israel isolate).

Rojas *et al.* (1993) standardised PCR method for the detection and differentiation of whitefly transmitted geminivirus using degenerate primers. PCR primers were tested for their effectiveness in the amplification of viral DNA fragments from the DNA-A and DNA-B components of 15 previously uncharacterized geminiviruses and obtained consistent amplification with the primer combination of PALlv1978 and PARlc496.

Degenerate oligonucleotide primers for the detection of whitefly transmitted geminiviruses were designed by Deng *et al.* (1994) and these primers amplified 500

bp fragment of DNA-A of five well characterized whitefly transmitted geminivirus by PCR. Thirteen geminivirus isolates from the leaf samples and six viruses from single viruliferous whiteflies were detected and viruses were also distinguished by the pattern of DNA fragments obtained with restriction endonucleases on the PCR products.

McGlashan *et al.* (1994) used PCR for the detection of TYLCV in the tomato Cv. Oxheart, grown under field conditions. The viral DNA got amplified when degenerate primers specific to genome A (PASIV 1978 and PARIC 496) and genome B (PBCIv 2040 and PCRC1) were used.

Khan (2000) could detect ToLCV both in its host *Lycopersicon esculentum* and vector *B. tabaci* using geminivirus specific degenerated primers in PCR. Muniyappa *et al.* (2000) standardised PCR detection of ToLCV using degenerated primers both from infected tomato plants and viruliferous *B. tabaci* and opined that PCR could be instrumental in studying the epidemiology of ToLCV and in the breeding of ToLCV resistant tomato varieties suitable to Bangalore region. Morris *et al.* (2002) reported PCR to be a more sensitive method than triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) for the detection of TYLCV isolates from all the hosts.

Reddy *et al.* (2005) carried out PCR using ToLCV coat protein specific primers (CRv 301 and CRc1152) and detected the presence of ToLCV in the symptomatic leaves. Reddy *et al.* (2011) found that, five representative symptomatic samples of ToLCV collected from the different regions of North-Karnataka were positive in polymerase chain reaction with the primer set ToLCBV33F and ToLCBV1070R and amplicons were obtained at 1040bp region.

PCR amplification of the genomic DNA extracted from virus infected plants was carried out by Thakuria *et al.* (2012) using ToLCV 2 primer pairs, yielded a 348bp fragment and confirmed the presence of ToLCV in the diseased plants.

The virus associated with tomato leaf curl disease in West Bengal, Sub-Himalayan Tarai region and Brahmaputra valley of Assam were detected by PCR using *Begomovirus* specific primers, DengA/ DengB (Saha *et al.*, 2014). Total fifty four diseased samples from fifteen locations were tested and all the samples showed positive PCR reaction, amplifying an expected size of 530 nt sequence fragment of CP gene of begomoviruses.

Rolling circling amplification (RCA) using the bacteriophage ϕ 29 DNA polymerase was reported to be comparatively easier and better method for a reliable diagnosis of geminiviruses (Haible *et al.*, 2006). Many workers have used this method for the complete genome extraction of geminiviruses (Kumari *et al.*, 2010; Pandey *et al.*, 2010; Rajasri *et al.*, 2011).

Knowledge of DNA sequences is indispensable for molecular characterization of plant viruses. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species plant pathogenic viruses.

The complete genome of a Thailand isolate of the geminivirus *Tomato yellow leaf curl virus* (TYLCV-Th) was cloned and sequenced. The results indicated that, genome consists of two DNAs each slightly greater than 2700 nucleotides in length and designated as DNA- A and DNA-B. Based on computer-assisted sequence comparisons with other geminiviruses, TYLCV-Th maintained the greatest degree of similarity with a tomato leaf curl virus isolated from Australia (Rochester *et al.*, 1994).

Padidam *et al.* (1995) sequenced the genomes of two isolates of *Tomato leaf curl virus* from India (ToLCV-India). ToLCV-India contains A and B components. The two isolates have 94 per cent sequence identity. The genome organization of ToLCV-India is similar to other whitefly-transmitted geminiviruses (WTGs) with bipartite genomes. Comparison of sequences and other biological features of ToLCV-

India with other geminiviruses showed that ToLCV-India is a distinct virus and is related to the WTGs from the Old World. ToLCV-Pan has a bipartite genome (DNAs A and B) and computer analysis showed that, the genome resembled that of other bipartite whitefly-transmitted geminiviruses. DNA-A (2584 nt) and B (2542 nt) have little sequence homology other than within the common region. Homology analysis of DNA A and B showed that ToLCV-Pan was most closely related to potato yellow mosaic virus (PYMV) from Venezuela (Engel *et al.*, 1998).

The degenerate DNA-A-specific PCR primers were used to amplify and clone the genome of a ToLCV isolate (ToLCV-Ban4) from Bangalore. The full-length of 2759 nucleotide long DNA-A like viral genome was sequenced. Sequence comparisons indicated that, ToLCV-Ban4 is similar to the other three isolates previously sequenced from Bangalore and is closely related to ToLCV-Ban2 (approximately 91% nucleotide sequence identity). Phylogenetic analysis showed that the ToLCV isolates from Bangalore constitute a group of viruses separated from those of north India (Muniyappa *et al.*, 2000).

Chatchawankanphanich and Maxwell (2002) did the comparison of the nucleotide sequence of cloned ToLCBV (pIND9) with other tomato associated begomoviruses from India and the results showed moderate DNA sequence identities (82 to 87 per cent) between capsid protein (CP) genes. Phylogenetic trees generated with nucleotide sequences of Rep and CP genes of 26 begomoviruses indicated that, this ToLCV was distinct from other begomoviruses and it might be a recombinant virus derived from at least three different viral lineages.

Chakraborty *et al.* (2003) cloned and sequenced DNA-A and DNA-B of components of two isolates collected from Varanasi and Mirzapur. Based on the viral DNA sequences, the new *Begomovirus* isolates were identified as *Tomato leaf curl Gujarat virus* and designated as ToLCGV(Var) and ToLCGV(Mir) respectively. The two genomic components of ToLCGV(Var) showed only 60% identity in the

common region. In addition, no other A or B components were found, which led to the conclusion that, the two components of ToLCGV(Var) were the cognate pairs that induced severe symptoms. DNA-B of ToLCGV(Var) showed 80 per cent sequence identity with that of ToLCNDV.

Replicase protein gene of three isolates of whitefly transmitted gemini virus causing leaf curl disease of tomato in India were amplified, cloned and sequenced by Praveen *et al.* (2004). Nucleotide sequence and the derived amino acid sequence for the replicase gene of these isolates were analyzed and phylogenetic relationship indicated as two subsets, one belonging to *Tomato leaf curl virus* having bipartite genome and the other having the monopartite genome.

Rojas *et al.* (2005) have characterised the complete nucleotide sequences of the DNA-A and DNA-B components for the first time for *Tomato leaf curl Sinaloa virus* (ToLCSinV). In addition, the complete nucleotide sequence was determined for the DNA-A component of two isolates of *Tomato severe leaf curl virus* (ToSLCV). The genome organization of ToLCSinV and ToSLCV were identical to the bipartite genomes of other begomoviruses described from America. Phylogenetic analysis of the DNA-A and DNA-B components and their open reading frames indicated that ToLCSinV and ToSLCV belong to different clades: ToLCSinV to the AbMV clade, and ToSLCV to the SLCV clade.

Reddy *et al.* (2005) amplified, cloned and sequenced the coat protein (CP) genes from 29 samples and the phylogenetic analysis revealed that, the CP sequences were grouped in five different tomato leaf curl *Begomovirus* clusters, with less than 88 per cent identity to each other. Four clusters represented known Indian ToLCVs, whereas one cluster contained sequences originating from Haryana with most identity (89 per cent) to the provisional *Begomovirus* species *Croton yellow vein mosaic virus*.

Reddy *et al.* (2011) analyzed four CP sequences obtained from the samples of ToLCV collected from the different regions of North-Karnataka. Phylogenetic trees were generated for the four CP sequences together with representative sequences available in gene bank and the four isolates clusterd into two groups. The isolates had highest homology of 92.40-96.00 per cent with ToLCBV-AVT 1 (AY 428770) and proved that, these isolates were entirely different from north Indian isolates and there was some variability within the isolates collected from a geographical location, indicating that there will be continuous variability in gemini viruses.

Thakuria *et al.* (2012) checked the sequence similarity of ToLCV-Jorhat isolates with different ToLCV isolates available in the database and the nucleotide sequence exhibited maximum 90 per cent similarity to ToLCPV – Pakistan, followed by 88 per cent to ToLCGV- Gujarat. However, in the dendrogram generated with the sequences from NCBI database using Tajima's coefficient of similarity ToLCV-Jorhat isolate grouped with ToLCV-Ranchi.

Saha *et al.* (2014) obtained seven CP sequences of ToLCV from the samples collected from W. Bengal and Assam and the phylogenetic analysis showed two groups amongst all the isolates analyzed. The isolates COB-2, RAI-1 and SILIGURI-2 showed 94-99 per cent sequence identity with different isolates of *Tomato leaf curl New Delhi virus* (ToLCNDV) and others showed similarity to *Tomato leaf curl Ranchi virus* (ToLCRaV) and *Ageratum enation virus* (AEV).

The novel isolates of ToLCV-K3, ToLCV-K5 from Kerala and ToLCV-CTM from New Delhi were characterized from the RCA products by Pandey *et al.* (2010). They reported that, ToLCV-K3 and ToLCV-K5 isolates might be the strains of the same virus since they showed sequence homology of 97 per cent over their entire genome. ToLCV-CTM and ToLCV-K3/K5 viruses were found to be monopartite as neither DNA-B component nor betasatellite were detected in these species. The complete nucleotide sequence of DNA-A genome of ToLCV-CTM exhibited highest

sequence homology (88 per cent) to *Croton yellow vein mosaic virus* (AJ507777), and of isolates K3/K5 (88.5 per cent) to *Tomato leaf curl Pakistan virus* (DQ116884). The sequence analysis and phylogenetic results proved that the genomes of these isolates exhibited multiple putative recombination events among themselves and had integrated other pieces of DNA that might have presumably originated from other viruses not identified so far. Furthermore, their study also demonstrated the wide sequence variability among ToLCV isolates from India.

Briddon *et al.* (2003) cloned and sequenced 26 additional DNA β molecules, associated with diverse plant species obtained from different geographical locations, and their findings strongly supported the co adaptation of DNA β molecules with their respective helper begomoviruses. Kumari *et al.* (2010) cloned and characterized DNA-A and DNA-β from ToLCV infected tomato plants from Patna, and reported a new monopartite *Begomovirus* and a betasatellite from north India and suggested the name *Tomato leaf curl Patna virus* (ToLCPaV). Phylogenetic dendrogram, based on alignment of complete DNA-A sequences of the majority of tomato-infecting begomoviruses of Indian sub-continent origin and other begomoviruses occurring in Asia showed that, ToLCPaV did not cluster with any other tomato infecting *Begomovirus* species originating from India rather it was placed on a separate clade with other tomato infecting *Begomovirus* species from Laos and Malaysia. Phylogenetic diagram of DNA-β showed that ToLCPa beta was placed in the same clade along with other betasatellites associated with tomato infecting begomoviruses isolated from Bangladesh and Pakistan.

ToLCV samples from Hyderabad were subjected to RCA and the products were cloned and sequenced (Rajasri *et al.*, 2011). ToLCV samples of Hyderabad, shared a maximum identity of 93 per cent with three Indian ToLCV strains *viz.* ToLCNDV-AVT1 from New Delhi (AY428769), ToLCNDV-Svr (U15015) and ToLCNDV-Chilli (EU309045). They reported that, complete nucleotide sequence of the Hyderabad isolate was found to be identical to *Tomato leaf curl New Delhi virus*

(ToLCNDV) strain when it was compared with other ToLCV known sequences in the NCBI GenBank through BLAST analysis.

2.9. Disease management

Efforts to manage tomato leaf curl disease have been made since the early phase of plant protection as this disease causes severe damage to tomato crop. Effects of cultural practices, plant protection chemicals, botanicals and bioagents in the management of disease have practical importance and scientific interest. Many workers have conducted extensive studies on these aspects.

The manipulation of the planting date to avoid coincidence of peak population of vectors would effectively reduce the chances of infection. Tomato cultivation during autumn and winter season in north India and during summer in south India was adversely affected with ToLCD (Sastry *et al.* 1978; Butter and Rataul, 1981; Saikia and Muniyappa, 1986) as compared to the other seasons.

Singh (1989) and Reddy (2006) noted that, the spread of ToLCD was reduced by the use of barrier crops such as maize, sorghum, and pearl millet. Another study conducted by Sastry *et al.* (1978) revealed that, intercropping with fast growing crops like maize, sunn hemp, pearl millet could reduce the incidence of leaf curl disease in tomato. Mulching with sawdust, fresh wheat straw or yellow polythene sheet reduced *B. tabaci* population and the incidence of ToLCD in Israel (Cohen and Madjar, 1978; Cohen *et al.* 1974; Cohen and Berlinger, 1986). Krishnareddy (1989) monitored whitefly population on black gram with the yellow sticky trap and it was found useful than other methods.

The protection of nurseries using physical and chemical methods against the vector found to reduce the incidence of ToLCD (Ioannou, 1985: Nakhla and

Maxwell, 1998). Tripathi (2000) found that, covering plants with perforated polythene bags in the initial stages, reduced the disease incidence.

The scope of regular use of insecticides is limited due to the cost and adverse environmental hazards. In recent years, naturally occurring substances in plants with antiviral properties have been recognized and tested against a wide range of viruses infecting various crops by many workers.

Verma and Verma (1993) reported two fold increase in nodulation and 50 per cent reduction in mungbean yellow mosaic incidence when dry leaf powder of *Clerodendron aculeatum* was applied as soil drench in addition to six times foliar spray at weekly interval.

Baranwal and Ahmad (1997) observed that foliar spraying coupled with soil application of aqueous solution of 1:8 dilution of *C. aculeatum* powder had better effect in delaying and reducing ToLCV incidence.

Louis and Balakrishnan (1996) noted 80 per cent reduction of pumpkin mosaic virus infection with the application of 10 per cent plant extracts of *Basella alba, Glycyrrhiza glabra, Phyllanthus fraternus, Plumbago rosea and Thespesia populnea.*

Pun *et al.* (1999) obtained 83.3 and 81.7 reduction in okra yellow vein mosaic incidence with leaf extracts of *Prosopis chilensis* and *Bougainvillea spectabilis* respectively. Louis (2003) noted that, spraying of *Plumabgo* extracts reduced pumpkin mosaic symptoms and application of plant extracts at shorter intervals was found to be effective in suppressing disease symptoms.

Bajpai (2005) obtained better management of chilli leaf curl virus with combined application of commercial botanical viricides, Pre-vental B.V/ Action 100 (0.2 per cent) with imidacloprid (1ml/3ltr) by reducing the incidence to 6 and 5 per cent respectively against 89 per cent in control. Reddy (2006) observed suppression

of tomato leaf curl disease with *Clerodendron inermis* and *Gliricidia* leaf extract at the early stage of the crop (45 DAT) with 13.37 and 14.50 per cent incidence as compared to 48.33 per cent in control.

Arunakumara *et al.* (2010) observed reduction in tomato early blight disease with 0.1 and 0.2 per cent Perfekt. Meena (2012) reported the efficacy of Perfekt in preventing the growth and sporulation of *Alternaria pori,* causing purple blotch of onion under *in vitro* conditions and also noticed reduction in disease incidence of 28.29 per cent against 72.09 per cent in control.

Karthikeyan *et al.* (2009) obtained 90 per cent reduction of leaf crinkle disease in black gram with the leaf extracts of *Mirabilis jalapa* and *Bougainvillea spectabilis*. Extracts of *Thuja orientalis*, *Tamarix brachystachy*, and *Lawsonia inermis* had exhibited inhibitory effects on TYLCV multiplication in tomato treated plants with protection periods of 10 to 12 days (Al-ani *et al.*, 2011).

The application of 3.5 % neemazol, 2% neem oil and 5% NSKE were found superior in reducing the whitefly population in okra by Naik *et al.* (2012).

Samiyappan (2003) reported the efficacy of *Pseudomonas fluorescens* in suppressing the viral infection and inducing systemic resistance in tomato plants against whiteflies and thrips.

Mishra *et al.* (2012) screened fifty rhizobacterial isolates against tomato leaf curl virus (ToLCV) disease under glasshouse condition and found that application of rhizobacteria based bioformulations to seed, soil and foliage significantly reduced the disease severity from 85.72 to 28.58 per cent with two isolates of *Pseudomonas* sp. Mishra *et al.* (2014) reported that, application of chitin based *Pseudomonas* reduced the disease severity of 90.33 to 80.33 per cent and also observed that, addition of chitosan has enhanced the bioefficacy of *Pseudomonas* against ToLCV.

Cherian (1998) noted 88.8 per cent mortality of *B. tabaci* with *Verticillium lecanii* after 72 h inoculation under laboratory condition. She has also observed maximum mortality of whiteflies in *Beauveria bassiana* and *V. lecanii* treated plots under field conditions. *V. lecanii* and NSKE were reported to be effective in controlling the whiteflies in okra (Anitha, 2007). Chandrashekharaiah *et al.* (2013) noted that *V. lecanii* @ 2.5 kg/ha could reduce the whitefly population and resulted in 10.98 per cent increase in yield.

Averre and Gooding (2000) suggested that, spraying the tomato bed with whole or skim milk @ 0.5 gallon / 100 sqft bed and dipping hands in milk while handling plants, could reduce the virus disease incidence.

Treatment of tobacco mosaic virus (TMV) contaminated tools with 20 per cent solution of nonfat dry milk (NFDM) and 0.1per cent Tween-20 completely eliminated TMV transmission in *Petunia* (Lewandowski, *et al.*, 2010).

Abdelbacki *et al.* (2010) observed suppression of *Tomato yellow leaf curl virus* infection with the application of native or modified whey proteins fractions at 15 days of treatment.

A number of insecticides have been reported to be effective against whiteflies by various workers. It is beyond the scope of the present study to go through the extensive literature and the numerous insecticides tested against whiteflies and this review is therefore confined only to the insecticides tested understudy.

The incidence of ToLCV was reduced with the use of mineral oil sprays and also increased the yield of tomato (Butter and Rataul, 1973, Singh *et al.*, 1973; Sastry *et al.*, 1976). The application of insecticides such as dimethoate, as spray and phorate as soil application were quite effective in reducing the whitefly population, checking the ToLCD incidence and increasing the yield (Sastry and Singh, 1973, 1974; Sastry *et al.*, 1976; Rataul and Butter, 1976).

The chemicals like chlorinated hydrocarbons, organophosphates, neonicotinoids, pyridine-azomethines, and pyrethroids were reported to be effective against whitefly. But, whiteflies have developed resistance to many of these chemicals and efficacies had decreased over time (Ahmed *et al.*, 2001; Polston and Anderson, 1997).

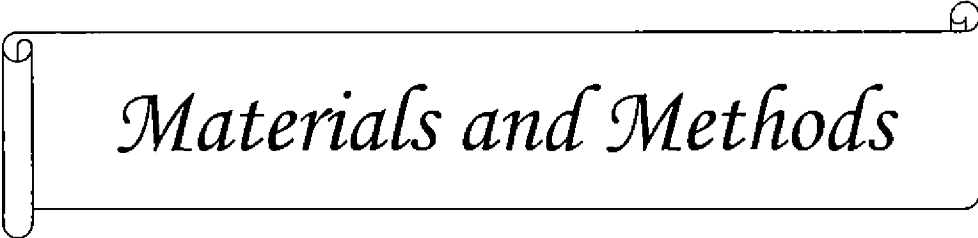
In addition to these insecticides, oils, insecticidal soaps, and insect growth regulators have also been used for controlling whiteflies. The most effective and widely used class of insecticides to reduce whitefly populations is the neonicotinoids of which thiomethoxam, imidacloprid, and dinotefuran have been widely used to reduce incidence of tomato leaf curl virus in many tropical countries (Ahmed *et al.*, 2001; Cahill *et al.*, 1996; Polston and Anderson, 1997, Polston and Lapidot, 2007).

Iersel *et al.* (2000) reported that, the application of imidacloprid to *Poinsettia* by subirrigation as a practical and effcient method to control silverleaf whiteflies. Zacharia (2006) observed reduction in the bittergourd distortion mosaic disease with imidacloprid 0.025 per cent. The application of imidacloprid 0.05 per cent at 15 days interval was found effective for the control of whitefly population to reduce the incidence of ToLCD (Reddy, 2006).

Ramu *et al.* (2011) carried out an experiment to manage *B. tabaci*, the vector of yellow vein mosaic of mesta and found that, acetamiprid (@ 0.2g/l) and thiamethoxam (@ 0.2g/l) were found most effective which recorded lowest whitefly population, disease incidence (3.15%) and highest fibre yield(18.00q/ha) and followed by imidacloprid (@ 0.2ml/l) and triazophos@ 2ml/l.

Chandrashekharaiah *et al.* (2013) found that, imidacloprid applied green gram plots recorded less whitefly population, yellow vein mosaic incidence and highest yield.

The effectiveness of carbendazim fungicide in the suppression of severity of TMV and lettuce western yellows disease has been already reported by Tomilson *et al.* (1976). Thomas and John (1980) also observed the suppression of tungro virus disease of rice plants with the application of carbendazim.


Materials and Methods

3. MATERIALS AND METHODS

The present study on "Characterisation and management of tomato leaf curl virus in Kerala" was carried out during 2012-2014. Experiments were carried out at the Department of Plant Pathology, College of Horticulture, Vellanikkara except serology and molecular works which were conducted at College of Agriculture, Vellayani and Banana Research Station, Kannara respectively.

3.1. Survey

A roving survey was conducted during September and October 2013 in tomato growing area of Kozhinjampara in Palakkad district. Second survey was conducted during March 2014 at Kozhinjampara, Vandithavalam, Perumatty, Vadakarapathy and Eruthempathy of the same district. Simultaneously, survey was also conducted in the research plots of College of Horticulture during the above periods. The disease incidence, severity and different types of symptoms were recorded from the surveyed areas. One hundred plants were selected from each plot for recording the incidence and severity.

Per cent disease incidence was calculated using the following formula

$$\text{Per cent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Disease severity was assessed by adopting a score chart of 0-4 scale as suggested by Banerjee and Kalloo (1987)

0 : symptom absent

1 : very mild curling (upto25 per cent leaves)

Plate: 1 General view of tomato fields in surveyed areas

Palakkad

Vellanikkara

2 : curling, puckering of 26-50 per cent leaves.

3 : curling, puckering of 51-75 per cent leaves

4 : severe curling, puckering of >75 per cent leaves

Per cent disease severity (PDS) was calculated using the formula suggested by Wheeler (1969).

$$\text{PDS} = \frac{\text{Sum of numerical rating}}{\text{No: of plants observed} \times \text{Maximum disease grade}} \times 100$$

Based on the per cent disease severity (PDS) and per cent disease incidence (PDI), the coefficient of infection (C I) was calculated using the formula (Datar and Mayee, 1981)

$$\text{Coefficient of infection} = \frac{\text{Per cent disease incidence} \times \text{Per cent disease severity}}{100}$$

3.1.1. Meteorological data of Vellanikkara

Weather parameters such as maximum and minimum temperature, relative humidity, and rain fall during the survey period in Vellanikkara were recorded.

3.2. Symptomatology of the disease

The development of the symptoms on leaves, flowers and fruits at different stages of plant growth were observed under natural and artificial conditions. Based on observations, symptoms were codified.

3.3. Studies on transmission of ToLCV

Graft and vector transmissions were conducted to prove the viral etiology of the disease. The studies were conducted in the insect proof net house during November 2013 to March 2014.

3.3.1. Graft transmission

Both wedge and approach grafting methods were adopted for the transmission of ToLCV.

3.3.1.1. Wedge grafting

Scions were collected separately from the diseased plants showing four different types of symptoms and a few leaves were trimmed off to prevent transpiration. The basal portion of the stem was cut to a wedge shape. Top portion of the healthy root stock plant was cut off at the nodal region and a longitudinal slit was given to a length to accommodate the wedge portion of the scion. The scion was then inserted to the slit of the stock, tied with thread and wrapped with moistened cotton. Grafted plants were covered with moistened polythene cover to maintain humidity and kept in net house for symptom appearance (Plate – 2).

3.3.1.2. Approach grafting

Healthy tomato plants were raised in polybags for root stocks. The tomato plants showing four different types of symptoms were uprooted from field and

Plate:2 Graft Transmission

Approach grafting

Wedge grafting

planted separately in poly bags which served as scions. Root stock and scion having same stem thickness were selected and brought in intimate contact by removing a slice from the side portion of both scion and root stock. The cut should be preferably smooth and flat. The cut surfaces were bound and tied together with thread and covered with moistened cotton. The grafted plants were covered with moistened polythene covers and were kept in net house for symptom appearance (Plate – 2).

3.3.2. Vector transmission

Vector transmission study was conducted using the whitefly, *Bemisia tabaci.*

3.3.2.1. Rearing / inoculation cages

Rearing / inoculation cages were designed with four sided metal frame covered with muslin cloth, having a small window at the front side covered with a polythene flap for the collection / release of whiteflies (Plate - 3)

3.3.2.2. Maintenance of whitefly culture

The whiteflies were collected from the brinjal fields of Department of Olericulture, using a glass test tube. The collected whiteflies were reared on healthy brinjal plants raised in earthern pots, kept inside the insect proof rearing cages of size 35 x 35 x 75 cm (Plate - 3). Old plants were replaced periodically with healthy young plants for the proper maintenance of the whitefly culture.

3.3.2.3. Collection of whiteflies

Whiteflies were collected from the rearing cages for the transmission studies using an aspirator, which was designed with Tarsons tube, rubber tube, rubber cork, glass tubes (Plate- 3).

Plate: 3 Whitefly Transmission

Whitefly rearing cage

Whiteflies on brinjal leaves

Aspirator for collecting whiteflies

Cage for acquisition of virus

3.3.2.4. Cages for the acquisition of virus from source plants

Plastic water bottles of size 27 x 7 cm were used for making the cages. The base and neck portion of bottles were removed using a sharp knife. One end of the bottle was covered with muslin cloth for aeration and other end kept free for covering the plants. A hole was made on the side of the cage for the release of whiteflies which was plugged with a cork (Plate - 3).

3.3.2.5. Source of inoculum

ToLCV infected twigs showing four different types of symptoms were collected from the research fields of Department of Olericulture and planted separately in plastic containers containing moistened soil which served as the source of virus inoculum.

3.3.2.6. Test plants

20 day old tomato seedlings raised in black polybags were kept in inoculation cages @ 10 / cage. Separate cages were kept for each type of symptoms.

3.3.2.7. Acquisition and inoculation access periods.

Whiteflies were collected from the rearing cages with the help of aspirator and released separately to the caged diseased twigs showing different types of symptoms @ 50/cage. After 24 h of acquisition access period, the viruliferous whiteflies from concerned virus source were released onto healthy tomato seedlings kept in inoculation cages separately. The plants were disturbed periodically for the distribution of the whiteflies to all plants. After 48 h of inoculation access period, the inoculated plants were sprayed with insecticide, dimethoate to kill the whiteflies. Cages were removed and the plants were kept for symptom development.

Transmission studies were conducted periodically to maintain the virus source plants for the molecular and serological studies

3.3.3. Transmission with whiteflies collected directly from ToLCV infected field

Whiteflies were collected directly from the infected plants showing three types of ToLCV symptoms. No whiteflies could be obtained from the plants showing yellowing and curling type of symptom as the number of such infected plants were less than five. The collected whiteflies were released onto healthy tomato seedlings for inoculation access period. Inoculated plants were kept in net house for symptom appearance.

3.4. Serodiagnosis

Serological experiments were conducted at the Protein Lab, Department of Plant Pathology, College of Agricuture, Vellayani. Direct Antigen Coating Enzyme Linked Immuno Sorbent Assay (DAC-EISA) suggested by Barbara and Clark (1982) was used for the serological studies. As the antisera of either ToLCV or TYLCV was not available the serological studies were conducted with *Indian cassava mosaic virus* (ICMV), *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) adopting DAC- ELISA methods. Details of the antisera used are given in Table- 1.

Fresh, younger leaves of both healthy and infected leaves were collected from the plants maintained in the net house in separate cages. Four samples of ToLCV infected plants with distinct symptoms and one healthy plant sample were used in two replications.

3.4.1.1. Reagents used

1. Stock buffer (phosphate buffer saline 1x PBS, pH-7.4) (Appendix- I)
2. Coating buffer: carbonate buffer, pH 9.6 (Appendix- I)
3. Wash buffer (PBS-Tween, PBS-T)

Table -1. Details of the antisera used for serodiagnosis

Sl.No.	Antisera	Type	Source	Dilution
1.	*Indian cassava mosaic virus* (ICMV)- SER 58	Monoclonal	IITA, Ibadan	1:200
2.	*African cassava mosaic virus* (ACMV)	Polyclonal	IITA, Ibadan	1:200
3.	*Sri Lankan cassava mosaic virus* (SLCMV)	Monoclonal	DSMZ, Germany	1:50

4. Enzyme conjugate diluent /buffer (PBS-T poly vinyl pyrrolidone ovalbumin, PBS- TPO). Added 20.0 g poly vinyl pyrrolidone (PVP-MW 44,000) and 2g egg ovalbumin to one litre PBS-T
5. Antibody diluents /buffer- same as PBS-TPO
6. Substrate buffer (diethanol amine buffer, pH- 9.8) Diluted 97 ml diethanol amine in 800 ml distilled water, pH adjusted to 9.8 using 6N hydrochloric acid and made upto one litre.
7. Blocking solution (Added 5g Bovine serum albumin to one litre PBS-T)
8. Stop solution (Sodium hydroxide 12g was dissolved in distilled water and made upto one litre).

3.4.1.2 Methodology

1. Leaf samples of 0.5g were weighed and the five samples were ground separately in pre chilled pestle and mortar with 2.5 ml buffer (0.4g PVP was dissolved in 20 ml of the buffer).
2. The ground samples were transferred to Eppendorf tubes and labelled. It was subjected to centrifugation in Hettich Zentrifugen, Micro 24 - 48r at 5000 rpm for 15 min.
3. Added 100µl supernatant to the wells of labelled microtitre plate. The plates were covered and incubated at 37^0 C for 1 h.
4. Washed the plate in PBS-T three times. Finally removed the remaining drops of washing buffer by gently tapping plates upside down on a tissue paper.
5. Added 100µl of blocking solution to each well and incubated at 37^0 C for one hour to block the unoccupied sites.
6. Washed the plates with PBS-T as before.
7. Added 100µl crude antiserum (primary antibody) diluted in PBS-TPO to each well. Incubated at 4^0 C overnight.
8. Washed the plates with PBS-T as before.

9. Added 100µl enzyme labelled (alkaline phosphatase) antirabbit IgG (secondary antibody) diluted in PBS-TPO to the wells which were coated with ACMV, ICMV and SLCMV. Incubated at 37^0 C for 2 h.
10. Washed the plates three times as before.
11. Added 100µl freshly prepared substrate, para nitrophenyl phosphate (0.5mg/ml) to each well. Incubated at room temperature for 30 min. in dark.
12. The reaction was stopped by adding 50µl of 3M sodium hydroxide to each well.
13. Measured absorbance at 405 nm in an ELISA reader (Microplate Manager Bio-Rad, Laboratories, Inc.)

3.5 Molecular characterisation

Studies on molecular characterization of the virus were carried out in the Molecular virology laboratory of Banana Research Station, Kannara.

3.5.1 Isolation of DNA

For the isolation of DNA, tender leaves of the ToLCV infected plants maintained in the net house, were collected in the early morning hours. The tender leaves of four different types of symptoms were collected separately in ice box. The leaf surface was cleaned by washing with sterile water and wiping with 70 per cent ethyl alcohol.

DNA isolation was done adopting the method suggested by Rogers and Bendich (1994) with slight modification (Method I). Modified protocol suggested by Doyle and Doyle (1990) was also tried to isolate the DNA with minimum phenol interference (Method II).

Reagents used:

a. CTAB buffer (2X):
   - 2 per cent CTAB (W/V)
   - 100 mM Tris base (pH 8.0)
   - 20 mM EDTA (pH 8.0)
   - 1.4 M NaCl
   - 1 per cent Poly vinyl pyrrolidon (PVP)
   - 0.2 per cent 2-β Mercaptoethanol
b. Chloroform: Isoamyl alcohol (24:1 v/v)
c. Chilled Isopropanol
d. Wash buffer
   - 76% Ethyl alcohol
   - 10 mM Ammonium acetate
e. Absolute alcohol
f. TE buffer:
   - 10 mM Tris (pH 8)
   - 1 mM EDTA (pH 8)
g. Sterile distilled water

Reagent a. and f. were autoclaved and stored at room temperature.

Composition of reagents is provided in Appendix II

3.5.1.1. Method I

1. One gram of sample was ground in pre-chilled mortar and pestle in the presence of liquid nitrogen. One ml of extraction buffer (2X), 10μl of β-mercaptoethanol and a pinch of PVP were added to the mortar.
2. The homogenized sample was transferred into a sterile 50ml Oakridge tube and 5ml of pre-warmed extraction buffer was added.

3. The contents were mixed well and incubated at 65°C for 30 min. with occasional mixing by gentle inversion.
4. Added equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify.
5. Centrifuged at 12,000 rpm for 15 min. at 4°C (Thermo Scientific Heraeus Biofuge stratos centrifuge).
6. Transferred the top aqueous layer to an Oakridge tube, added 5ml of chloroform: isoamyl alcohol (24:1) and mixed by inversion.
7. Spun at 12,000 rpm for 15 min. at 4°C.
8. The supernatant was transferred into an Oakridge tube, added 0.6 volume of chilled isopropanol and mixed gently.
9. Centrifuged at 8,000 rpm for 5 min. at 4°C. Gently poured off the supernatant and saved the pellet.
10. Added 2.5 ml of absolute alcohol and spun at 8,000 rpm for 5 min. at 4°C.
11. The alcohol was poured off, the pellet was air dried for half an hour, dissolved in 50µl distilled water and stored at -20^{0}C.

3.5.1.2 Method II

1. Preheated 5 ml of CTAB buffer (2X) in 50 ml Oakridge centrifuge tube to 60°C in a water bath.
2. Fresh leaf tissue (0.5-1.0 gm) was ground with a pinch of polyvinyl pyrrolidin (soluble) and 50µl of 2 per cent 2-β-mercaptoethanol in 60°C CTAB buffer in a preheated mortar and pestle.
3. The samples were incubated at 60 °C for 30 (15-60) minutes with occasional gentle swirling.
4. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10,000 rpm for 10 min. at room temperature.

5. The content got separated into three distinct phases.

Aqueous top layer	-	DNA with small quantity of RNA
Middle layer	-	Protein and fine particles
Lower layer	-	Chloroform, pigments and cell debris

6. The aqueous phase transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by gentle inversions till the DNA got precipitated.
7. Centrifuged at 10,000 rpm for 3 min. at 25^0C and discarded the supernatant.
8. Added 1ml of wash buffer (76 % ethanol +10mM ammonium acetate) and incubated at room temperature for 20 min.
9. Centrifuged at 10,000 rpm for 10 min. at 25^0C and remaining solution was poured off.
10. The pellet was air dried and dissolved in 30µl distilled water and stored at - 20^0C.

DNA was isolated separately from leaves showing four different types of symptoms. DNA from the leaves of healthy plants was also extracted which served as control.

3.5.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of the DNA.

Reagents used:

1. Agarose - 0.8 per cent (for genomic DNA)
   - one per cent (for PCR product)
2. 50X TAE buffer (pH8.0)

3. Tracking/loading dye (6X)

4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml)

Composition of reagents is provided in Appendix III

Procedure

1. The gel caster was placed on a horizontal surface. The comb was placed properly in gel caster.
2. Prepared 0.8 per cent agarose (0.8 g in 100ml) in a conical flask with 100 ml 1X TAE buffer. Micro waved for 45 to 60 sec. until agarose was completely dissolved and the solution was clear.
3. Solution was allowed to cool to about 42 to 45^0 C before pouring. 2µl ethidium bromide was added at this point to a concentration of 10 µl/ml and mixed well.
4. Poured this gel solution into the gel tray and allowed the gel to solidify for about 30 to 45 min. at room temperature.
5. To run, gently removed the comb, placed the tray in buffer tank, and submerged (just until wells were submerged) with electrophoresis buffer (1X TAE).
6. To prepare samples for electrophoresis, mixed 1 µl of tracking dye with 5µl of DNA solution. Mixed well and loaded 6µl DNA sample per well. Loaded suitable molecular weight marker (λDNAEcoRI) in one lane.
7. The cathode and the anode of the electrophoresis unit were connected to the power pack and the gel was run at constant voltage of 70 volts until the dye has migrated two third the length of the gel.

3.5.3. Gel documentation

The DNA bands separated by electrophoresis were viewed and documented using Bio-Rad Gel Doc EZ Imager.

3.5.4. Purity of DNA

The purity of DNA was further assessed by using Nano drop 2000C (Thermo scientific) spectrophotometer. Nucleic acid shows absorption maxima at 260nm where as proteins show peak absorbance at 280nm. Absorbance was recorded at both wavelength and purity was indicated by the ratio OD_{260}/OD_{280}. The quantity of DNA in the sample was calculated using the relation 1 OD_{260} equivalent to 50µg double stranded DNA/ml sample.

3.5.5 Polymerase chain reaction

The DNA of ToLCV was amplified from the total host DNA using PCR (Eppendorf Master Cycler Gradient). Emerald Amp® GT PCR mastermix (Takara Bio Inc. Japan) was used for the present study. PCR mix (total 50µl) was prepared as per the manufacturer's guidelines.

Emerald Amp® GT PCR mastermix	- 25µl
Template	- 37 ng
Forward primer	- 0.2µM (final concentration)
Reverse primer	- 0.2µM (final concentration)
Distilled water	- up to 50µl

PCR assay was carried out using primers which are already reported. The details of the primer are furnished in Table- 2. These primers are specific to the coat protein region.

3.5.5.1. Standardization of PCR conditions

Various combinations of PCR parameters like annealing temperature and template dilutions were tested to find out the optimum combination of conditions. The temperature gradients were set to find out the optimum annealing temperature for the PCR. Annealing temperatures of 57- 63^0C were tested for ToLCBV33F and ToLCBV1070R and 48-54^0C for CRv301 F and CRc1152 R. Different template concentrations of 15ng/µl, 37ng/µl, 75ng/µl and 376.7ng/µl were used for the standardization of PCR.

50µl of reaction mixture was dispensed in 0.2 ml tubes and was given a momentary spin for thorough mixing of the cocktail components. The PCR tubes were then placed in thermal cycler (Eppendorf Master Cycler Gradient). The PCR was carried out with two sets of primers as furnished in Table - 3.

The amplicons were electrophoresed in one per cent agarose gel, documented and compared with 1kb DNA marker (Genei, Bangalore).

3.5.6 Elution of amplicons and sequencing

The gel piece containing amplified DNA fragment was carefully cut from the agarose gel under UV-transilluminator. The DNA fragment was eluted from the gel piece using Nucleospin® Gel and PCR clean up kit (Machery- Nagel GmbH & Co. KG, Germany) as per manufacturer's guide lines.

The eluted DNA fragments were sequenced at Sci Genome Labs, CSEZ, Kochi.

Table- 2. Details of the primers used in PCR assay

Sl.No.	Primer	Sequence	Expected product size	Reference
1.	ToLCBV 33F ToLCBV1070R	5'GGT CCC CTC CAC TAA ATCAT 3' 5' CAG TTG GTT ACA GAA TCG TAG AAG 3'	~1040bp	Reddy *et al.* (2011)
2.	CRv301 F CRc1152 R	5'ATGKCSAAGCGWCCRGCAGA 3' 5'TTWARAATGTAAWWKGAGCAG 3'	~870 bp	Reddy *et al.* (2005)

* K=G+T, R=A+G and W=A+T

Table- 3. Amplification conditions of PCR primers

Steps followed	Conditions for different primer sets			
	ToLCBV 33F ToLCBV1070R		CRv301 CRc1152	
Step1:Initial denaturation	94^{0}C - 2 min.		94^{0}C - 1 min.	
Step 2: Denaturation	94^{0}C - 45sec.	35 cycles	94^{0}C - 50 sec.	35 cycles
Step 3: Annealing	55^{0}C – 1min.		51^{0}C - 45 sec.	
Step 4: Extension	72^{0}C - 1.30 min.		72^{0}C - 1.30 min.	
Step 5: Final extension	72^{0}C - 20 min.		72^{0}C -10 min.	
Step 6: Cooling	4^{0}C – 5 min.		4^{0}C – 5 min.	

3.5.7 Comparison of ToLCV sequences

In silico analysis was carried out to compare the four sequences obtained from ToLCV plants showing different types of symptoms. The coat protein sequences of ToLCV isolates from various geographical locations were retrieved from NCBI. Megablast (www.ncbi.nlm.nih.gov/blast/blast.cgi) was carried out and the isolates showing maximum identity were selected for the comparison. Genebank searches were done using the BLAST program (Morgulis *et al.*, 2008). Dendrogram and phylogenetic analysis was carried out using the programme Mega 4 (Tamura *et al.* 2007).

The programme 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used to find the open reading frame of the four nucleotide sequences. Open reading frames available in the entire region was noted, saved and BLASTp search was also performed.

3.6 Management of tomato leaf curl disease

A field experiment was laid out during January to April 2014 at College of Horticulture, Vellanikkara, to find out the effect of botanicals, chemicals and bioagents on the management of tomato leaf curl disease.

Experiment details

Variety	: Anagha
Design	: Randomised Block Design
Replication	: 3
Plot size	: 2.7m x 1.2 m
Spacing	: 45 cm x 60 cm

Treatments : 17

Number of plants per treatment : 12

3.6.1 Nursery preparation

Tomato seedlings of Anagha were raised in earthern pots containing sterilized potting mixture consist of soil, sand and cow dung @1:1:1. The pots were kept in net house and irrigated regularly.

3.6.2 Preparation of land

Experimental plot was prepared by ploughing followed by leveling. Shallow trenches were taken at a length of 2.7 m and width of 30 cm and 20 day old seedlings were planted at a spacing of 45 x 60 cm. Agronomic practices were adopted as per KAU, Package of Practices Recommendations Crops- 2011.

3.6.3 Treatments

Treatments were given as three foliar sprays. First spray was given at 15 days after planting, and subsequent two sprays were given at 15 days interval. Imidacloprid is an yellow labelled systemic insecticide that, only two sprays were given, as the plants were in fruiting stage at the time of third spray. Treatments details are shown in Table - 4.

3.6.4 Observations

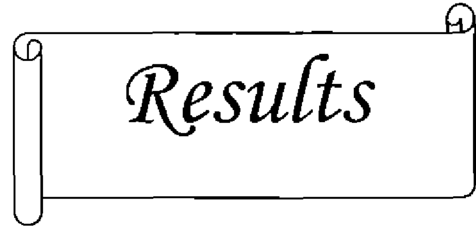
Observations on type of symptoms, disease incidence and severity were recorded at 10 days after each treatment. Biometric observations like plant height, days to flowering, days to fruiting, number of fruits per plant and yield were recorded

Table- 4. Details of treatments

Treatments	Treatment details
T_1	Imidacloprid 17.8 SL-25g ai ha^{-1} (0.3ml/l)
T_2	Neem oil – 2% (20ml/l)
T_3	*Gliricidia maculata* leaf extract 10% (100ml/l) + cow milk 1% (10ml/l)
T_4	*Clerodendron infortunatum* leaf extract 10% (100ml/l) + cow milk 1% (10ml/l)
T_5	*Lawsonia inermis* (Henna) leaf powder 0.4% (4g/l) + cow milk 1% (10ml/l)
T_6	Perfekt-0.2% (2ml/l)
T_7	Imidacloprid 17.8 SL-25g ai ha^{-1} (0.3ml/l)+ Perfekt 0.2%
T_8	Action 100- 0.2% (2ml/l)
T_9	Imidacloprid 17.8 SL-25g ai ha^{-1}(0.3ml/l) + Action 100- 0.2%
T_{10}	*Pseudomonas fluorescens* - 2% (20g/l)
T_{11}	*Pseudomonas fluorescens* -2% + Perfekt-0.2%
T_{12}	*Pseudomonas fluorescens* -1% (10g/l) + carbendazim 0.1% (1g/l)
T_{13}	*Verticillium lecanii* - 0.5% (5g/l)
T_{14}	Curd- 10% (100ml/l)
T_{15}	Yellow sticky trap @2/ plot , 60 cm distance
T_{16}	Lime -0.5% (5g/l) + turmeric powder -1%(10g/l)
T_{17}	Control

3.5.5. Statistical analysis

Data was analysed following analysis of variance for randomized block design (Gomez and Gomez, 1984). Multiple comparisons among treatment means, where the F test was significant was done with Duncan's Multiple range test using MSTAT package. The data was transformed if necessary and statistically analysed.


Results

4. RESULTS

The results of the investigation carried out on tomato leaf curl virus (ToLCV) disease during 2012 – 2014 are presented in this chapter.

4.1. Survey

A roving survey was conducted in farmers' field at Kozhinjampara, Vandithavalam, Vadakarapathy, Perumatty, Vadakarapathy and Eruthempathy, the major tomato growing areas of Palakkad district to record the incidence, severity and type of symptoms of leaf curl disease during the months of September 2013, October 2013 and March 2014. The results are presented in Table – 5.

During the survey conducted in September, in the two locations of Kozhinjampara, where the crops were in seedling and fruiting stages, the disease incidence and severity were comparatively less, recording 2 – 5 and 2 – 2.8 per cent respectively. In October, a slight increase in infection was noticed at the first location where the crop reached to fruiting stage. During these periods, only upward curling and cupping and curling and rolling type symptoms were observed in these locations, of which latter was more predominant.

In March, the crops were in early/late fruiting stages and showed severe infection of leaf curl in all surveyed locations, recording 74 to cent per cent incidence and 61.25 – 83.75 per cent severity. Except yellowing and curling, the other three types of symptoms were observed during this period, of which, upward curling and cupping type were more prevalent, showing 22 – 72 per cent incidence followed by curling and rolling type (16 – 58 per cent) and only 6 – 21 per cent plants showed curling and purple tint symptom.

Survey conducted at Vellanikkara also showed less infection during October to December, recording 22 – 47 per cent incidence and 13.75 – 26.25 per cent disease

Table – 5. Survey on the occurrence of tomato leaf curl disease in Palakkad district

Season	Location	Hybrid variety	Stage of the crop	PDI	PDS	Type of symptom (per cent incidence)			
						C	R	P	Y
September 2013	Kozhinjampara								
	Location 1	Meera	Seedling	2	2.00	0	2	0	0
	Location 2	Meera	Fruiting	5	2.80	1	4	0	0
October 2013	Kozhinjampara								
	Location 1	Meera	Fruiting	6	3.30	1	5	0	0
March 2014	Kozhinjampara	Meera	Fruiting	100	81.25	72	28	0	0
	Vandithavalam	Sivam	Fruiting	90	70.00	22	58	10	0
	Perumatty	Sivam	Fruiting	82	66.25	23	38	21	0
	Vadakarapathy	Meera	Fruiting	81	83.75	54	21	6	0
	Eruthempathy	Lakshmi 5005	Fruiting	74	61.25	58	16	0	0

C-Upward curling and cupping,

R- Curling and rolling

P- Curling with purple tint

Y- Yellowing and curling

severity and both found increasing in the subsequent months, reached to 83 per cent incidence and 67.63 per cent severity during the month of March, in the variety Anagha. All the four types of symptoms were prevalent in this area throughout the period. Among these, upward curling and cupping and curling and rolling type were most predominant recording 8 – 33 per cent and 6 – 28 per cent incidences respectively. Curling with purple tint symptom was noticed 5 – 18 per cent. Yellowing and curling type symptoms were rare and observed only 3 – 4 per cent (Table - 6).

Thus, from the survey it was observed that, ToLCV infection was severe during summer months as compared to rabi season. Upward curling and cupping and curling and rolling types were the most common symptoms observed in the surveyed areas. It was also observed that, the hybrid varieties, Meera, Sivam, Lakshmi grown in Palakkad areas and bacterial wilt resistant variety, Anagha grown at Vellanikkara were highly susceptible to ToLCV.

The meteorological data presented in Table – 7 for the period from September 2013 to March 2014 showed that, the mean temperature varied from 26.3 to 28.2^{0} C during rabi and 27.95 to 30.45^{0} C during summer season with maximum (30.45^{0} C) in the month of March. The mean relative humidity was also high (61 – 85 per cent) during the rabi season as compared to summer (51 – 55 per cent). It is also observed that there was no rainfall during summer months. Thus this data showed that, the high temperature, less humidity and rainfall favoured the high incidence of ToLCV.

4.2. Symptomatology

Symptoms of ToLCV were studied under both natural and artificial conditions.

Table – 6. Survey on the occurrence of tomato leaf curl disease in Vellanikkara campus

Season	Stage of the crop	PDI	PDS	Type of symptom (per cent incidence)			
				C	R	P	Y
October 2013	Vegetative	22.0	13.75	8.0	6.0	5.0	3.0
November 2013	Vegetative	38.0	20.00	13.0	14.0	8.0	3.0
December 2013	Fruiting	47.0	26.25	15.0	16.0	12.0	4.0
January 2014	Fruiting	64.0	37.75	22.0	26.0	12.0	4.0
February 2014	Fruiting	75.0	53.17	28.0	27.0	16.0	4.0
March 2014	Fruiting	83.0	67.63	33.0	28.0	18.0	4.0

C-Upward curling and cupping

R- Curling and rolling

P- Curling with purple tint

Y- Yellowing and curling

Table – 7. Meteorological data of Vellanikkara during the period September 2013 to March 2014

Parameter	September 2013	October 2013	November 2013	December 2013	January 2014	February 2014	March 2014
Mean temperature (^{0}C)	26.60	27.35	28.20	27.65	27.95	28.80	30.45
Mean Relative Humidity (%)	85	83	73	61	51	56	55
Rain fall (mm)	344.1	369.8	82.0	0.5	0	0	0

4.2.1 Symptom observed under natural conditions

The general symptoms of the disease were curling and puckering of the leaves, stunted growth, reduction in leaf size and intermodal length resulted in bushy appearance. The flowers were completely or partially sterile. The infected plants produced few fruits and no fruits depend on the stage of the crop at infection. Apart from the above general symptoms four unique types of symptoms were also recorded which are detailed below.

4.2.1 Symptom observed under natural conditions

Four types of symptoms were observed on ToLCV infected plants in Kerala and the details of the symptoms are described below (Plate- 4).

4.2.1.1. Type I- Upward curling and cupping

- Stunted growth
- Leaves turned dark green and vein banding in some cases
- Leaf margin curled inward cup like appearance
- Upward bending of petioles
- Puckering and smalling of younger leaves
- Symptoms on younger plants
    - Severe stunting
    - Excessive branching and led to bushy appearance.
    - Flowering was very rare
    - Fruit set was also rare and fruits were smaller if formed
- Symptoms on older plants
    - Younger leaves showed cupping and older leaves slight curling
    - Internodal length reduced
    - Clustering of apical portion

- Flower abscission
- Fruits were less
- Fruit size reduced

4.2.1.2. Type II- Curling and rolling

- Leaves curled inward and rolled
- Drooping of leaves along with petiole
- Downward bending of petioles
- Leaves turned pale in some plants
- Leaves became leathery
- Younger leaves elongated and tips became pointed
- Leaf veins turned purple in some cases,
- Younger plants
    - Stunted growth
    - No flowering
- Older plants
    - Yield was not affected

4.2.1.3. Type III- Curling with purple tint

Symptoms were similar to curling and rolling (Type- II). The unique symptom of this type was the appearance of purple tint on the entire leaf lamina. In severe cases, entire plant appeared as a purple bush. Fruit formation was very less as compared type II.

4.2.1.4. Type IV- Yellowing and curling

- Yellowing and inward curling of leaves.
- Leaves had papery texture in some plants

Plate: 4. Symptomatology

Under natural condition

Type I-Upward curling and cupping

Type II- Curling and rolling

Type II- Curling and rolling

Type III- Curling with purple tint

Type IV- Yellowing and curling

- Younger leaves reduced in size and became pointed
- Margin of certain younger leaves showed yellowing
- Older leaf petiole were bent downward
- Flowering was rare
- If fruits developed , they were small and yellowish
- Young plants
    - Stunting
    - Reduced leaf size with cupping and yellowing
    - No flowering

4.2.2 Symptoms observed under artificial conditions

Four types of symptoms associated with tomato leaf curl disease were studied under artificial conditions. Same types of symptoms were observed in both natural and artificial conditions. However, no flowering was observed in any of the inoculated plants under artificial conditions due to early infection (Plate 5).

4.2.2.1. Type I- Upward curling and cupping

In this type, symptom initiated as cupping, at 15 – 16 days of inoculation. Slight curling of the leaf margin was observed at 4 – 5 days of initial symptom.

4.2.2.2. Type II- Curling and rolling

Symptom appeared as a slight curling of the leaves after 17 days of inoculation. Typical rolling was observed six days after symptom appearance. Reduction in the leaf size and internodal length resulted in bushy appearance.

Plate: 5. Symptomatology

Under artificial condition

Type I-Upward curling and cupping

Type II- Curling and rolling

Type III- Curling with purple tint

Type IV- Yellowing and curling

4.2.2.3. Type III- Curling with purple tint

Symptom noticed as slight curling of the leaf margin at 14 – 16 days of inoculation and purple tint appeared 5 days after the initial symptoms.

4.2.2.4. Type IV- Yellowing and curling

Initial symptom appeared 14 – 18 days after inoculation. The initial symptom was slight curling of the leaf margin with marginal yellowing. As the disease progressed, the yellowing spread to entire leaf lamina.

4.3. Studies on transmission of ToLCV

Both grafting and vector transmission studies were adopted to ascertain the infectious nature of tomato leaf curl disease.

4.3.1. Transmission of ToLCV by grafting

Wedge grafting and approach grafting were carried out with the scions of four different types of symptoms. Data presented in the Table – 8 showed that, per cent transmission and incubation period varied with the grafting methods adopted. Among the two methods tried, approach grafting showed higher rate of transmission and early appearance of symptoms. This method showed 50 – 70 per cent transmission and 14 – 17 days incubation period against 10 – 30 per cent and 24 – 28 days in wedge grafting. It is also noted that, per cent transmission varied with the type of symptoms also. Yellowing and curling type and upward curling and cupping types of symptoms showed high per cent transmission of 70 and 30 with approach and wedge grafting respectively.

4.3.2. Transmission of ToLCV by whitefly

The whitefly transmission experiments carried out during October to December 2013 (rabi) and January to March 2014 (summer) showed, high rate of

Table – 8. Transmission of ToLCV by grafting

Sl.No.	Type of symptom	Wedge grafting		Approach grafting		Remarks
		Mean per cent transmission	Incubation period (Days)	Mean per cent transmission	Incubation period (Days)	
1.	Upward curling and cupping	30	25	60	15	
2.	Curling and rolling	10	27	60	17	
3.	Curling with purple tint	10	28	50	14	Purple tint appeared five days after the initial symptom appearance
4.	Yellowing and curling	20	24	70	14	

transmission and less period of incubation in summer as compared to rabi season. During October – December the transmission per cent was 45 – 60 and the incubation period was found to be 27 – 33 days, whereas in January – March, transmission rate was high, recording 40 – 90 per cent and symptoms appeared in 16 – 18 days of inoculation. In both seasons, transmission rate and incubation period varied with the type of symptoms also. Highest transmission rates 70 and 60 per cent were observed in curling and rolling and upward curling and cupping type symptoms during summer and rabi respectively. However, less incubation period was observed in case of curling with purple tint in both seasons (Table – 9).

4.3.3. Transmission with whiteflies collected directly from the ToLCV infected field

Transmission study conducted with the whiteflies directly collected from the field, showed typical symptoms of ToLCV on test plants which indicated that the whiteflies collected from field were already viruliferous. In this case also, transmission rate was quite high, ranged from 60 – 70 per cent and symptoms appeared in 14 – 16 days of inoculation. Maximum transmission (70 per cent) was obtained with curling and rolling type. It was also noticed that, whiteflies collected from the plants of a particular symptom, showed the same type of symptom on the test plants except, in case of curling and rolling type, where typical rolling symptom was not evident as observed under natural condition (Table – 10).

Table – 9. Whitefly transmission of ToLCV

Sl.No.	Type of symptom	October –December 2013		January- March 2014	
		Mean per cent transmission	Incubation period (Days)	Mean per cent transmission	Incubation period (Days)
1.	Upward curling and cupping	60	30	80	16
2.	Curling and rolling	45	33	90	17
3.	Curling with purple tint	55	27	60	17
4.	Yellowing and curling	45	28	40	18

Table –10. Transmission studies with whiteflies directly collected from infected field

Sl.No.	Type of symptom	Mean per cent transmission	Incubation period (Days)	Type of symptoms produced
1.	Upward curling and cupping	65	14	Typical curling and cupping
2.	Curling and rolling	70	16	Upward curling only
3.	Curling with purple tint	60	16	Typical curling with purple tint

4.4 Serodiagnosis

DAC-ELISA method was adopted for serodiagnosis, using the antisera of three geminiviruses *viz. Indian cassava mosaic virus* (ICMV), *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV). ToLCV infected samples of tomato with four types of symptoms showed positive reaction with ACMV indicating that, it is serologically related to ACMV (Plate – 6). The mean value of absorbance at 405 nm varied (0.7625 – 1.0095) with samples and highest was recorded in curling and rolling type symptom. Absorbance at 405 nm is given in Table – 11.

4.5 Molecular characterisation

4.5.1 Isolation of DNA and Agarose gel electrophoresis

DNA was isolated from healthy as well as from infected plants with two different protocols. Among the two methods adopted, good quality DNA with less phenols and RNA contamination was obtained with the protocol given by Rogers and Bendich (1994) with slight modifications. Cream colored DNA pellets obtained when subjected to agarose gel electrophoresis, showed intact single band (Plate –7). Hence this method was found suitable for the extraction of DNA of ToLCV infected plants.

4.5.2. Estimation of quality and purity of DNA

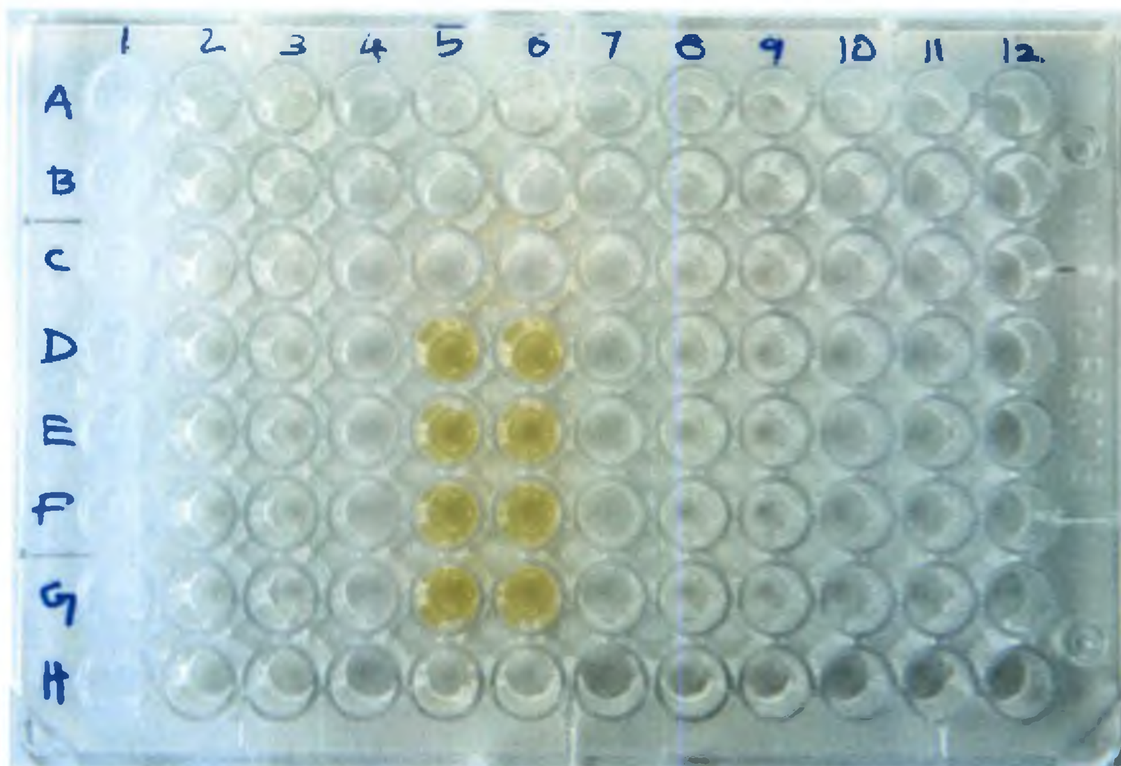
Quantities of DNA present in the infected samples were determined using nanodrop. The ratio of optical density value at 260 nm to that at 280 nm ranged from 1.73 to 1.90, indicating relatively pure DNA without protein contamination (Table – 12). The $OD_{260/280}$ value was highest for curling with purple tint (1.9) and least for yellowing and curling type (1.73).

Table – 11 Serological reaction of ToLCV to different antisera

Sl. No.	Sample	Absorbance at 405 nm		
		ICMV	ACMV	SLCMV
1.	Healthy	0.11	0.006	0.0075
2.	Upward curling and cupping	0.009	0.7625	0.0155
3.	Curling and rolling	0.008	1.0095	0.016
4.	Curling with purple tint	0.0075	0.8365	0.013
5.	Yellowing and curling	0.0625	0.826	0.0175

ICMV- *Indian cassava mosaic vius*, ACMV- *African cassava mosaic virus*, SLCMV- *Sri Lankan cassava mosaic virus*

Plate: 6. Serodiagnosis of ToLCV (DAC- ELISA reaction)



2 & 3 – Antisera of *Indian cassava mosaic virus*

5 & 6 – Antisera of *African cassava mosaic virus*

8 & 9 – Antisera of *Sri Lankan cassava mosaic virus*

B- Blank

C- Healthy

D-Curling & rolling

E- Upward curling and Cupping type

F- Curling with purple tint

G- Yellowing and curling

Table – 12. Quality and quantity of DNA synthesized

Sl. No.	DNA sample	OD $_{260/280}$	Quantity of DNA ng/µl
1.	Healthy	1.94	934.6
2.	Upward curling and cupping	1.84	376.7
3.	Curling and rolling	1.81	93.7
4.	Curling with purple tint	1.90	750.1
5.	Yellowing and curling	1.73	353.9

The quantity of DNA assessed by spectrophotometry using nanodrop varied from 93.7 – 750.1 ng/µl for various samples. The quantity of DNA was found to be high in curling with purple tint and was the lowest in curling and rolling type.

4.5.3. PCR detection of ToLCV

PCR amplification of ToLCV was carried out with already reported two sets of primers *viz*. ToLCBV 33F and ToLCBV1070R (Reddy *et al.*, 2011) and CRv301 and CRc1152 (Reddy *et al.*, 2005).

4.5.3.1. Standardisation of PCR conditions

PCR conditions such as annealing temperature and template dilutions were standardized.

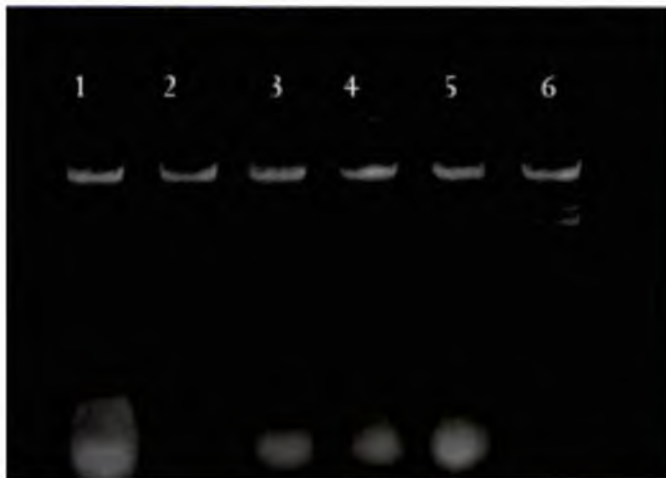
4.5.3.1.1. Effect of annealing temperature on PCR amplification

Annealing temperatures ranging from 57 – 63^{0}C were tried for the PCR amplification with the primers ToLCBV 33F and ToLCBV1070R and 48-54^{0}C were tried for CRv301 and CRc1152. Expected amplification was not obtained with the primers ToLCBV 33F and ToLCBV1070R, instead primer dimer was observed. In the case of CRv301 and CRc1152 primers, bands were observed between 800 and 900 base pairs. The intensity of amplification was highest at 51^{0}C and no amplifications were obtained at 53 and 54^{0}C (Plate – 7).

4.5.3.1.2. Effect of template dilution on PCR amplification

DNA sample with a concentration of 376.7ng/µl was diluted to 15ng/µl, 37ng/µl and 75ng/µl and used in PCR. Bands were not obtained with the primers ToLCBV 33F and ToLCBV1070R at any of the concentrations while PCR amplification with primers CRv301 and CRc1152 produced good bands at the template concentration of 37ng/µl (Plate – 8).

Plate: 7. Molecular detection of ToLCV



Lane 1- Upward curling and cupping type

Lane 2- Curling and rolling type

Lane 3- Curling with purple tint type

Lane 4- Yellowing and curling type

Lane 5- Healthy sample

Lane 6- 1 kb DNA Marker

DNA Isolation

Lane 1- 1kb DNAMarker

Lane 2- Healthy sample

Lane 3- 48^{0}C

Lane 4- 49^{0}C

Lane 5- 50^{0}C

Lane 6- 51^{0}C

Lane 7- 52^{0}C

Lane 8- 53^{0}C

Lane 9- 54^{0}C

Standardisation of annealing temperature

Plate: 8. Molecular detection of ToLCV

Lane 1- 1 kb DNA Marker

Lane 2- Healthy sample

Lane 3- 15 ng/µl

Lane 4- 37 ng/µl

Lane 5- 75 ng/µl

Lane 6- 376 ng/µl

Standardisation of template concentration

Lane M- 1 kb DNA Marker

Lane C- Upward curling and cupping type

Lane R- Curling and rolling type

Lane P- Curling with purple tint type

Lane Y- Yellowing and curling type

Lane H- Healthy sample

PCR detection of ToLCV in plant samples with four different types of symptoms

4.5.3.1.3 PCR amplification with ToLCV coat protein primers in plant samples with different types of symptoms

Polymerase chain reaction was carried out with two set of primers, with the DNA isolated from infected plants showing four different types of symptoms. All the four samples gave positive reaction with the primers CRv301 and CRc1152. A distinct band was observed between 800 and 900 base pairs in all the four samples (Plate – 8).

4.5.4 Elution and sequencing of PCR products.

PCR products of different types of symptoms were eluted, sequenced and sequence data was obtained in FASTA format (Table – 13). In upward curling and cupping type (Type I) 561bp and in curling and rolling (Type II) type 847 bp were obtained whereas 632 bp and 707bp were obtained in curling with purple tint (Type III) and in yellowing and curling type (Type IV) respectively.

4.5.5 Comparison of ToLCV sequences

The four sequences obtained for the different types of symptoms were compared, checked for homology. The sequences were found to be different at varying levels.

The phylogenetic diagram was constructed (Fig. – 1). The upward curling and cupping type (Type I) and curling and rolling type (Type II) came under the same clad whereas curling with purple tint (Type III) and yellowing and curling (Type IV) types formed another group. This indicated that the four different types of symptoms are incited by viruses which are different with respect to their coat protein region.

The sequences were separately subjected to megablast in NCBI and the sequences showing significant alignments with each type were selected and downloaded fromthe NCBI database (first five). Phylogenetic trees were constructed

Table – 13 Sequence data of eluted PCR products

Sl. No	Isolate	Size (bp)	Sequence (5'- 3')
1.	Upward curling and cupping (Type I)	561	GACAAAATTTTAGGCAGTATTAATCATATGTTGTATGTTGACAATCACATTCAATAAAAT TCAATATTTATTAATTCGATACAGAATCATAAAAATAGATCCGTATCTTCAATGTAGCAT ACACAGGATTAGAGGCGTGCGTAGACGCCATATACAACATCAAAGCATTCTCACTATGGT TCTCATACTTCCCAGCCTCTTGCTGGTTATAAACAACATAATTATTAACCCTAACAAACTT CTTCACCAAAGCCTGCTCCTTGGACGCATAGGTTCCACCAGTGACAGTTGCGTGCCACTT CCTCAATACTTGGTAGCGATCACGATGAACATTCTTCACGGTTGCAGTACTGGGCTCATT GTCAAACATATTAAACACCTCACCAAAATCCTGGGGTTTGTCAACAGGCCTCCTGTCACG ACACAAAAAAAACATCACACTATTCGTGTGATTCTTAGTCTTGATATTCTCATCCATCCAC ACCTTGCCCAATACATATACGGACTTAACACAAAACCTCTTACCGACCCTATGAGTAAGA CCAATTCCACGGGTGACAT
2.	Curling and rolling(Type II)	847	GCTAAAGAAAAAAGCATGTGATGCGACACACAGTGTATGTACAGAGTTCAAACATAATA AAATTTAATATTTATTAATTCGATACCGAATCATAGAAATAGATCCGTATCTTCAAAGTA GCATACACAGGGTTAGAGGCATGAGTACATGCCATGTACAACATCAGAGCATTCTCTGTG TGATTTTCATATTTTCCAGCTTCCTGCTGGTTATACACAACATAATTGTTAACCCTAACGA ACTTCTTAACTAATGCCTGTTCCTTCGAGGCGTACTGTCCTCCAGTAACAGTTGCGTGCCA TTTCCTCAAGACCTGGTAACGATCCCTATGCATATTCTTCACAGTCGCCGTGCTAGGCTCG TTGTCAAACATGTTGAACACCTCTCCAAAATCTTGTGGCTTATCAACAGGACGACGATCA CGTACAAGGAAGAACATCACACTATTCGTGTGATTCTTAGTCTTGATATTCTCATCCATCC ATATCTTCCCTAATACATACACAGACTTAACACAAAACCGTTTACCTACTCTATGGGTTA ACCCAGTACCACGGGTAACATCACTAATACACATGACCTTCCCTATATGAACTACATCGT GTCTAGACTCAAACGACTGGACCTTACATGGGCCTTCACATCCCCTTGGAACATCTGGGC TCCTGTACATCCTGTACATCCTGGGTTTCCGATTCATGGGCCTGTTGGCCCATGCTCTTGC CTTTGTGACGCGGACAATGGGGGCAGCAGCACGGCTGGCATATGGGCTGTCGAAGTTGA GACGGCGGCGTACCTTCGAAGCGGGCGTGGAAATGATTATATCTGCCGGTACGCTTCGCC CATA

3.	Curling with purple tint (Type III)	632	GCTTTTTTCATTTTCTTCGCATTGTACGCCGCCGTCTCCCTTCTTCAGCCCATATGTCATCC GTGCTGCTGTGCCATTGTGCGCGTCTAAAGTCAAGAGAATGGGGGAACACGCCCCTGTGC ACAAAACCCCCGATGTGTAGGATGTGCACAAACCCTGATGTCCCCCGAGGATATGAAAG CCCCTGTAAGGTACAATCATTTGAAAAAGGCATGATATTTCTCATATTGGGAAGGTCATG TGTAAAATGATGTTACTCGTGGGATTGGGCTAACTCATAAGGTTGGTAAACGTTTCTGTG TTAATCCCTTTAATGTTTGGGTAAAATATGGATGGATGAAAAATATCAGAACCAAAATCC CCCCAATAATGGTATGGTTTTTCCTGGTCCTGATCCTCCTCCTGGTTACCAACCCCAAAAC TTTTGAAAAGTGTTCCATATGTTTGACCATGAACCTAACACTGGTACTGGGAAAAAATGG CATCCAGATCCTTATCCAGGTTTGAAGAAATGGCATGGCACTGGCCATGGTGGTCCATAC CCTTCCAAAGGACAAGGATTAATGAAAAAATTGGTTAAGGTAAACATTATGGTTGTTAAT AACCCAAAAAGCTTGGAAAATAAAATTCCA
4.	Yellowing and curling (Type IV)	707	ATTTTTCCACCTGAATTCGCGTGTCGCCGCCGTCTCTCCTTCTTCAGCCCATATGTCATCC GTGCTGCTGCCCCCATTGTCCGCGTCTAAAGTCAAGAGCATGGGCGAACACGCCCCTGTA CACAAAACCCCCGATGTGCACGATGTACACAAACCCTGATGTCCCAAAAGGATATGAAA GCCCCTGTAAGGTACAATCATTTGAGCAAAGGCATGATATTTCTCTCACATTGGAAGGTC ATGTGTATATTATGATGTTACTCGTGGGATTGGGCTAACTCATAAGATTGGGTGCGTTTTT GTGTTAAATCCGTTTTACTTTTGGGTAAAAATGGATGGATGAAAATATTTAAACCAAAAA TCCACCAATAATGGTATGTTTTTTCCTGGTCCGATCCTCCTCCTGGTGACAAACCCAAAAC TTTTGAAAAGTGGTCAATATGTTTGAACATGAACCTAACACTGCTACTGGGAAAAATGGG CCATCAAATCCTTATCCAGTTTTGAAGAAAGGCATGGCACTGGTACTGGTGGTCCTACCC TTCCAAAGAACCAGGATTAATGAAAAATTGGTAAAGGTTAAAATTATGTTGTTTAAATCC ACCAGAAGGTTGGAAATATTAAAAACCTTCCGAGAATGCTTTTAAGGTTTTATAGGCATG GAATCCTTGCTCCAACCCGTGTAACCTAACTTAAAAATTCCTTTA

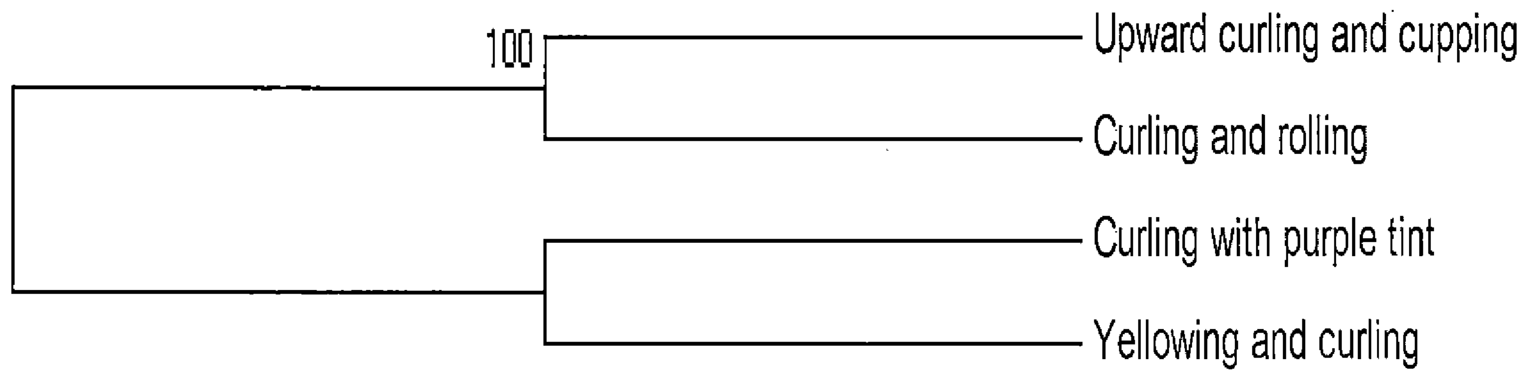


Fig – 1 Phylogram of four sequences

for four different types' sequences under investigation with the corresponding five sequences which showed maximum identity and query coverage in blast hits.

4.5.5.1 Sequence comparison of upward curling and cupping (Type I)

Homology search of nucleotide sequence of upward curling and cupping type with other reported begomovirus sequences were carried out. The sequences showed significant homology with different DNA- A / coat protein sequences of various species of *Begomovirus* present in NCBI data bank. This sequence showed query coverage of 98 per cent and identity of 96 per cent to *Ageratum yellow vein Sri Lanka virus* segment A (AF314144.1) with a maximum score of 911. In the blast hits it was followed by *Tomato leaf curl virus* AV1 (AJ810367.1) gene for coat protein with query coverage of 87 per cent and 98 per cent identity. Out of the 102 blast hits, upward curling and cupping type has shown maximum homology to *Ageratum yellow vein Sri Lanka virus* and *Tomato leaf curl virus* AV1 gene with minimum e- value (0.0). (Table – 14) (Plate – 9).

However, the phylogenetic analysis indicated upward curling and cupping (C) type has more evolutionary relation with ToLCV Karnataka isolates (HF563619.1 and HF563618.1) (Fig - 2).

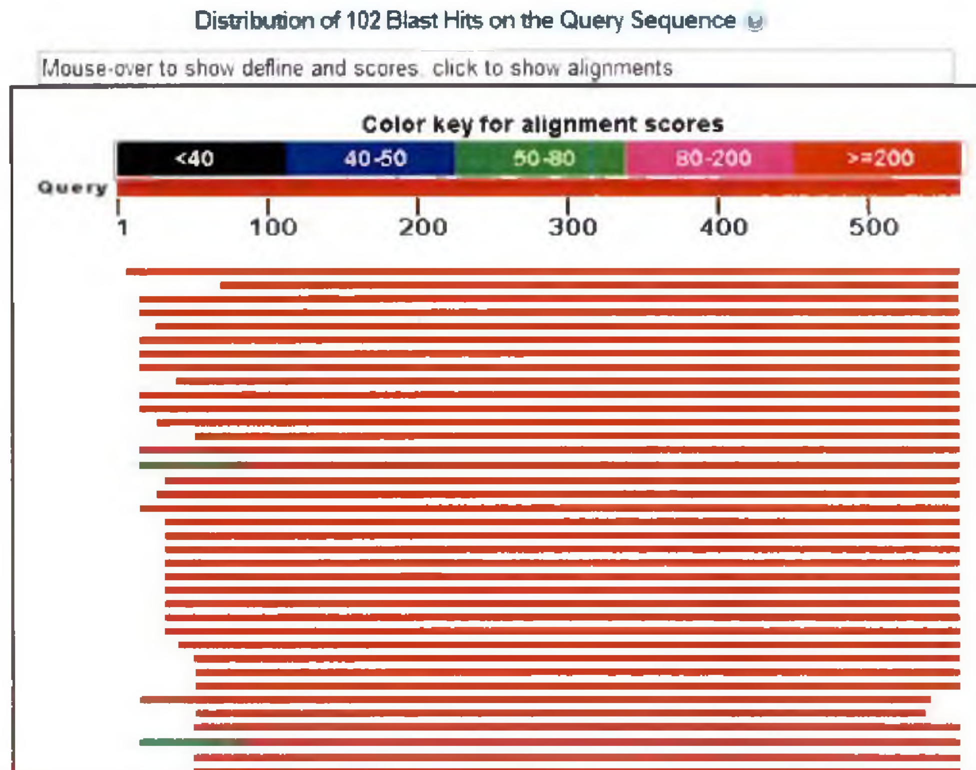
4.5.5.2 Sequence comparison curling and rolling type (Type II)

Result of blastn against curling and rolling type symptom indicated, 95 per cent query sequence showing 96 per cent homology with DNA-A of *Pepper (chilli) leaf curl Lahore virus* isolate (AM491589.1) with maximum score of 1303. This sequence also showed 94 per cent homology to *Tomato leaf curl virus* coat protein AV1(AY691901.1). Total hundred blast hits were obtained on the query sequence of curling and rolling type (Plate -10). However the curling and rolling type sequence has formed another branch when the dendrogram was constructed with the six sequences mentioned in Table – 15. (Fig –3). The results indicated that, curling and

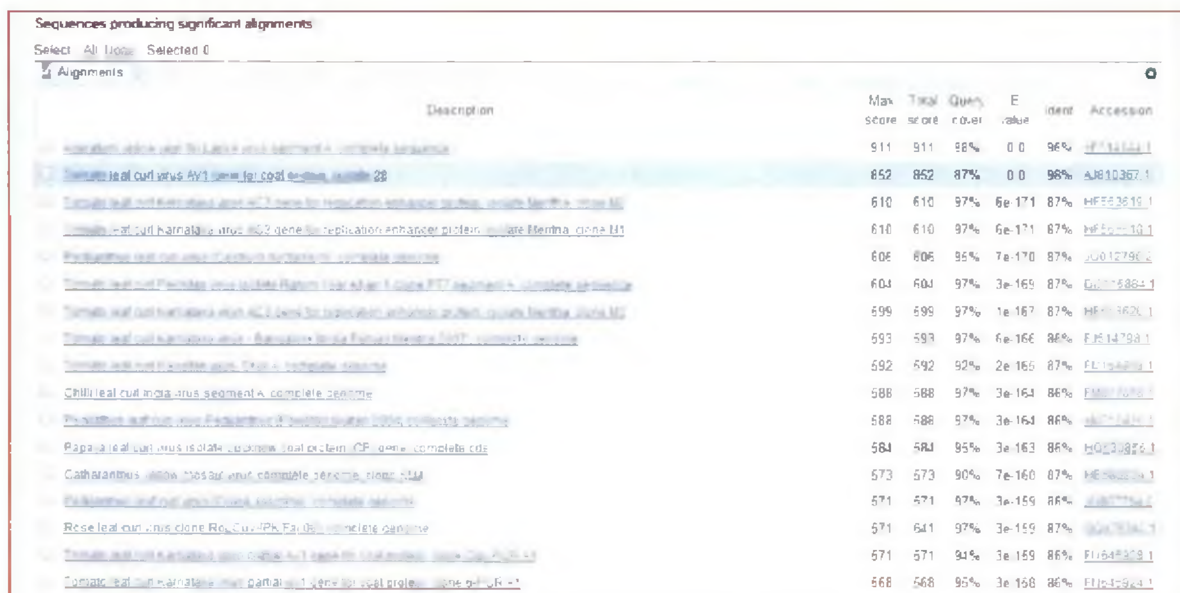
Table – 14. Sequence homology observed for upward curling and cupping type in BLASTn analysis as per BLAST results

Sl.No	Description	Max score	Total score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Ageratum yellow vein Sri Lanka virus* segment A, complete sequence	911	911	98.00	0.0	96.00	AF314144.1
2.	*Tomato leaf curl virus* AV1 gene for coat protein, isolate 28	852	852	87.00	0.0	98.00	AJ810367.1
3.	*Tomato leaf curl Karnataka virus* AC3 gene for replication enhancer protein, isolate Mentha, clone M2	610	610	97.00	5e-171	87.00	HF563619.1
4.	*Tomato leaf curl Karnataka virus* AC3 gene for replication enhancer protein, isolate Mentha, clone M1	610	610	97.00	5e-171	87.00	HF563618.1
5.	*Pedilanthus leaf curl virus*-[*Cestrum nocturnum*], complete genome	606	606	95.00	7e-170	87.00	JQ012790.2

Plate: 9. BLAST output of upward curling and cupping type



BLASTn graphical output



Sequences producing significant alignments

Select: All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
[illegible]	911	911	98%	0.0	96%	[illegible]
[illegible] leaf curl virus AV1 [illegible] coat [illegible] 28	852	852	87%	0.0	98%	AJ810367.1
[illegible]	610	610	97%	6e-171	87%	[illegible]
[illegible] leaf curl Karnataka virus AC3 gene for replication enhancer protein, isolate Mentha, clone M1	610	610	97%	6e-171	87%	[illegible]
[illegible]	606	606	95%	7e-170	87%	[illegible]
[illegible]	604	604	97%	3e-169	87%	[illegible]
[illegible]	599	599	97%	1e-167	87%	[illegible]
[illegible]	593	593	97%	6e-166	86%	FJ514798.1
[illegible]	592	592	92%	2e-165	87%	[illegible]
Chilli leaf curl India virus segment A, complete genome	588	588	97%	3e-164	86%	[illegible]
[illegible]	588	588	97%	3e-164	86%	[illegible]
Papaya leaf curl virus isolate [illegible] coat protein (CP) gene, complete cds	584	584	95%	3e-163	86%	[illegible]
Catharanthus yellow mosaic virus complete genome, clone [illegible]	573	573	90%	7e-160	87%	[illegible]
[illegible]	571	571	97%	3e-159	86%	[illegible]
Rose leaf curl virus clone [illegible] complete genome	571	641	97%	3e-159	87%	[illegible]
[illegible]	571	571	94%	3e-159	86%	[illegible]
Tomato leaf curl Karnataka virus partial [illegible] gene for coat protein [illegible]	568	568	95%	3e-158	86%	[illegible]

BLASTn text output

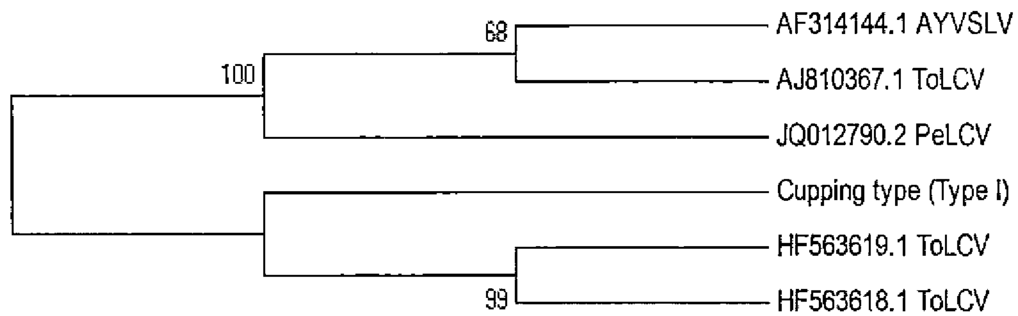


Fig – 2 Phylogram of upward curling and cupping type (Type I) with the sequences of top five blast hits

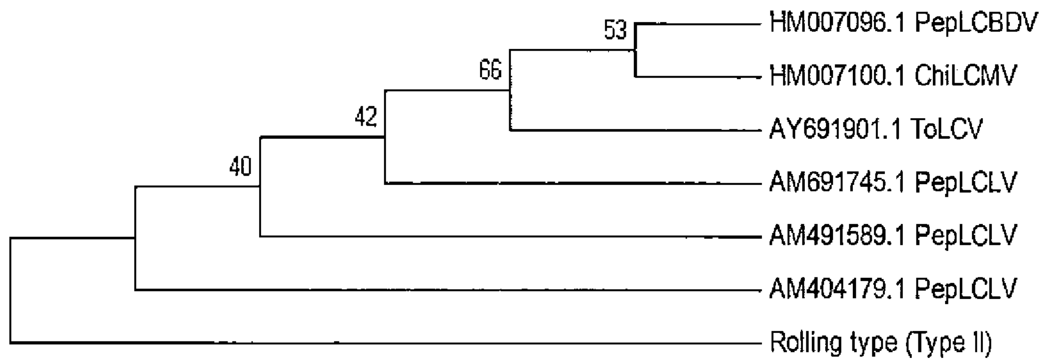
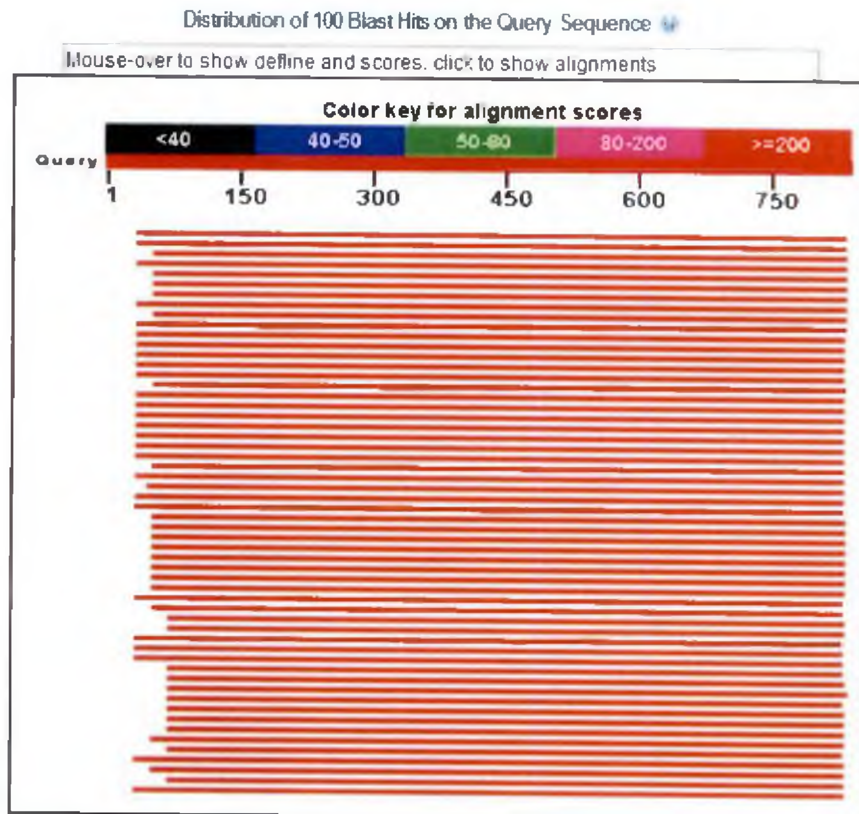


Fig: 3 Phylogram of curling and rolling type (Type II) with the sequences of six blast hits

Table – 15 Sequence homology observed for curling and rolling type in BLASTn analysis as per BLAST results

Sl.No.	Description	Max score	Total score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Pepper leaf curl Lahore virus*-[Pakistan:Lahore2:2004] segment A, complete sequence, clone PeAL2	1303	1303	95.00	0.0	96.00	AM491589.1
2.	*Pepper leaf curl Lahore virus* complete genome, clone P1	1297	1297	95.00	0.0	96.00	AM691745.1
3.	*Pepper leaf curl Bangladesh virus*-India [India/Coimbatore/2008] clone pChCoB2 segment DNA-A, complete sequence	1295	1295	92.00	0.0	96.00	HM007096.1
4.	*Pepper leaf curl Lahore virus*-[Pakistan:Lahore1:2004] segment A, complete sequence, clone BpAL 4(3)	1291	1291	95.00	0.0	96.00	AM404179.1
5.	*Chilli leaf curl Multan virus*-India [India/Guntur/2009] clone pChGuB16 segment DNA-A, complete sequence	1284	1284	92.00	0.0	96.00	HM007100.1
6.	*Tomato leaf curl virus* coat protein (AV1) gene, complete cds	1144	1144	90.00	0.0	94.00	AY691901.1

Plate: 10. BLAST output of curling and rolling type



BLASTn graphical output

Sequences producing significant alignments

Select: All None Selected 0

Alignments

	Description	Max score	Total score	Query cover	E value	Ident	Accession
	[illegible]	1303	1322	95%	0.0	96%	[illegible]
	[illegible]	1297	1297	95%	0.0	96%	[illegible]
	[illegible]	1295	1295	92%	0.0	95%	[illegible]
	[illegible]	1291	1291	95%	0.0	96%	[illegible]
	[illegible]	1284	1284	92%	0.0	96%	[illegible]
	[illegible]	1279	1279	92%	0.0	96%	[illegible]
	[illegible]	1279	1279	93%	0.0	96%	[illegible]
	[illegible]	1258	1258	94%	0.0	95%	[illegible]
	[illegible]	1247	1247	92%	0.0	95%	[illegible]
	[illegible]	1236	1236	95%	0.0	94%	[illegible]
	[illegible]	1221	1221	94%	0.0	94%	[illegible]
	[illegible]	1221	1221	94%	0.0	94%	[illegible]
	[illegible]	1216	1216	94%	0.0	94%	[illegible]
	[illegible]	1210	1210	94%	0.0	94%	[illegible]
	[illegible]	1205	1205	94%	0.0	94%	[illegible]
	[illegible]	1201	1201	92%	0.0	94%	[illegible]
	[illegible]	1199	1199	94%	0.0	94%	[illegible]
	[illegible]	1199	1199	94%	0.0	94%	[illegible]
	[illegible]	1199	1199	94%	0.0	94%	[illegible]
	[illegible]	1199	1199	94%	0.0	94%	[illegible]

BLASTn text output

rolling type symptom produced by ToLCV in Kerala is having maximum similarity with *Pepper (chilli) leaf curl Lahore virus*.

4.5.5.3 Sequence comparison curling with purple tint type (Type III)

Homology search of nucleotide sequence of curling with purple tint type gave six blast hits and it showed significant homology with DNA- A of *Tomato leaf curl Kerala virus* isolate TC-343 (KF551575.1)with 94 per cent query coverage, 82 per cent identity and maximum score of 486, followed by ToLCV K3 DNA-A (EU910141.1) and ToLCV K5 DNA-A (EU910140.1) with query coverage of 94 and 90 per cent and identity of 81 and 82 per cent respectively.(Table – 16) (Plate -11). The dendrogram was generated using the corresponding sequences and curling with purple tint type outgrouped with other five sequences (Fig – 4).

4.5.5.4 Sequence comparison yellowing and curling type (Type IV)

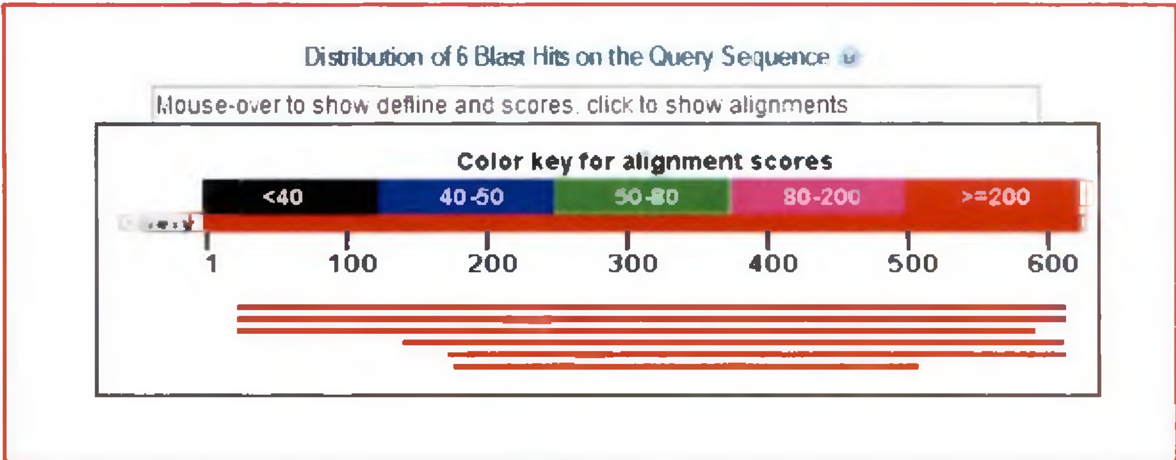
Analysis of similarity search of yellowing and curling sequence showed significant homology with DNA-A of three *Tomato leaf curl Kerala virus* isolates ToLCV K3 (EU910141.1), ToLCV K5 (EU910140.1) and ToLCV TC343 (KF551575.1) with 90 per cent query coverage and 82 – 83 per cent identity with maximum score 555. There were hundred blast hits for this sequence (Table – 17, Plate – 12). However, in the dendrogram for yellowing and curling type with other five sequences which has shown maximum similarity in the blast hits, the yellowing type out grouped with the other five sequences (Fig – 5).

Summing up the findings obtained during *in silico* analysis, it was observed that, all sequences were having maximum homology with DNA-A component of begomoviruses only. The upward curling and cupping and curling and rolling type inciting ToLCVs are different with respect to the coat protein sequences and are having maximum similarity with *Ageratum yellow vein Sri Lanka virus* DNA-A and *Pepper (chilli) leaf curl Lahore virus* DNA-A. Curling with purple tint and

Table – 16. Sequence homology observed for curling with purple tint type in BLASTn analysis as per BLAST results

Sl.No.	Description	Max score	Total score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Tomato leaf curl Kerala virus* isolate TC343 segment DNA-A, complete sequence	486	486	94.00	1e-133	82.00	KF551575.1
2.	*Tomato leaf curl Kerala virus* isolate ToLCV-K3 segment DNA A, complete sequence	481	481	94.00	5e-132	81.0	EU910141.1
3.	*Tomato leaf curl Kerala virus* isolate ToLCV-K5 segment DNA A, complete sequence	481	481	90.0	5e-132	82.00	EU910140.1
4.	*Alternanthera leaf curl virus* isolate Bengaluru coat protein (Cp) gene, partial cds	377	377	75.00	6e-101	81.00	JX827167.1
5.	*Alternanthera leaf curl virus* coat protein-like gene, partial sequence	351	351	70.00	4e-93	81.00	JQ693140.1

Plate: 11. BLAST output of upward curling with purple tint type



BLASTn graphical output

BLASTn text output

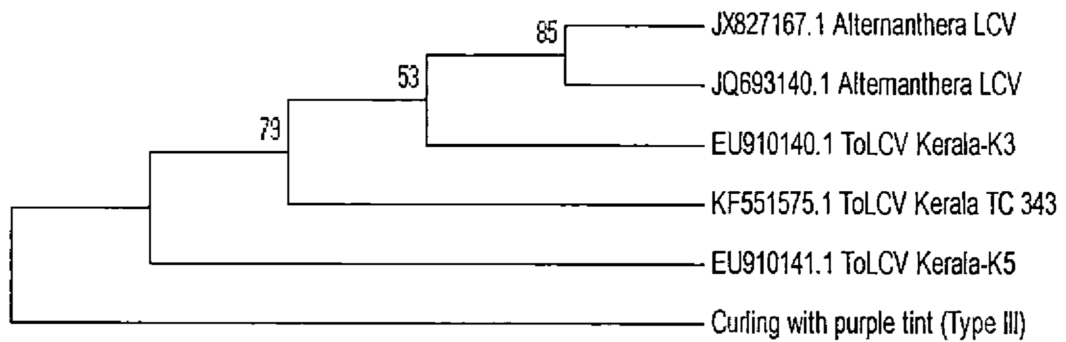


Fig: 4 Phylogram of curling with purple tint (Type III) with the sequences of top five blast hits

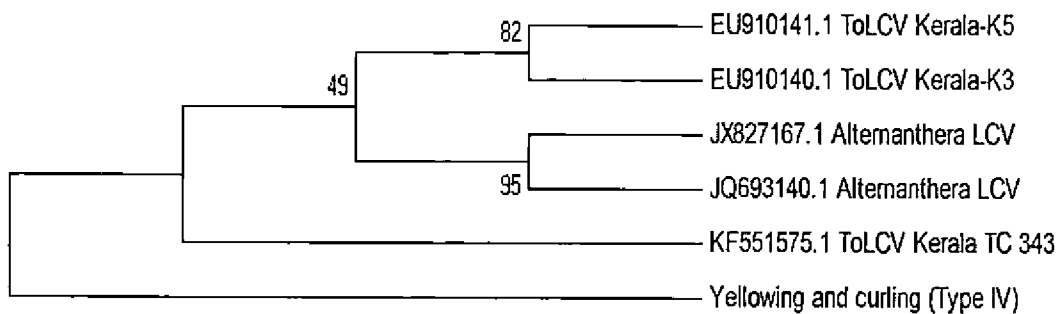
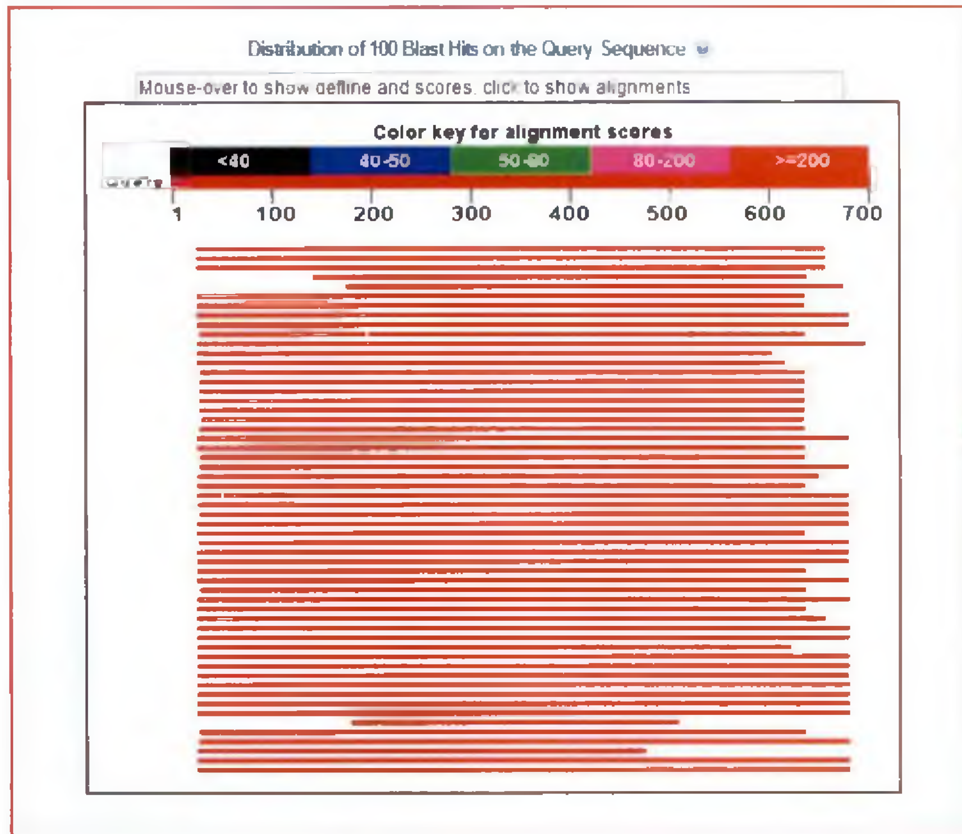


Fig: 5 Phylogram of yellowing and curling (Type IV) with the sequences of top five blast hits

Table –17 Sequence homology observed for curling andyellowing type in BLASTn analysis as per BLAST results

Sl.No.	Description	Max score	Total score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Tomato leaf curl Kerala virus* isolate TC343 segment DNA-A, complete sequence	555	555	90.00	3e-154	83.00	KF551575.1
2.	*Tomato leaf curl Kerala virus* isolate ToLCV-K3 segment DNA A, complete sequence	532	532	90.00	1e-147	82.00	EU910141.1
3.	*Tomato leaf curl Kerala virus* isolate ToLCV-K5 segment DNA A, complete sequence	412	412	90.00	1e-147	82.00	EU910140.1
4.	*Alternanthera leaf curl virus* isolate Bengaluru coat protein (Cp) gene, partial cds	387	387	70.00	2e-111	82.00	JX827167.1
5.	*Alternanthera leaf curl virus* coat protein-like gene, partial sequence	327	327	71.00	1e-103	81.00	JQ693140.1

Plate: 12. BLAST output of yellowing and curling type



BLASTn graphical output

BLASTn text output

yellowing and curling types were found similar to already reported ToLCV Kerala isolates. The four types of symptoms are found to be associated with begomoviruses only.

4.5.6. Detection of Open Reading Frame (ORF)

The programme 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used to find the open reading frame of the four nucleotide sequences. There were three open reading frames in upward curling and cupping type, with longest one located on +2 strand starting from base 35 to 560, having a length of 527 bases and the other two were located on -3 and -1 strands. Curling and rolling type had six open reading frames and the longest one was with a length of 627 bases coding 208 amino acid residues, starting from base 74 to 700 on -1 strand (Plate : 13). Seven ORFs were found in curling with purple tint type and the longest one was with a length of 255 bases, starting from 276 to 530. In the case of yellowing and curling type five ORFs were found with the longest one located on +3 strand starting from base 441 to 647, having a length of 207 bases (Table - 18). When the longest significant open reading frames of all four types were subjected to BLASTp search, all of them detected homology with coat protein of geminiviruses.

Table – 18. Open reading frames of coat protein gene from four isolates

Sl. No.	Isolate	ORF location	ORF length (bp)	Reading frame
1.	Upward curling and cupping	35-560	527	+2
		71-478	408	-3
		436-560	126	-1
2.	Curling and rolling	74-700	627	-1
		590-846	258	+2
		666-846	182	+3
		329-496	168	+2
		140-253	114	+2
		631-732	102	+1
3.	Curling with purple tint	276-530	255	-1
		8-226	219	-2
		1-201	201	-3
		54-251	198	+3
		441-631	192	+3
		337-507	171	+1
		479-580	102	+2
4.	Yellowing and curling	441-647	207	+3
		1-201	201	-3
		258-452	195	-1
		135-278	144	+3
		53-160	108	+2

Plate: 13. Open Reading Frame Finder output

Open reading frame (ORF) finder out put

BLASTp output of longest ORF

4.6. Management of tomato leaf curl disease

A field experiment was conducted to find out the effects of botanicals. bioagents, and chemicals on the management of tomato leaf curl disease. Observations were recorded on disease incidence and severity at 10 days after each spray and based on these, coefficient of infection was calculated.

4.6.1. Effect of treatments on ToLCV incidence.

From the data presented in Table – 19 it is found that, all treatments were superior to control after each spray. But, in general, none of the treatments gave complete protection to tomato leaf curl disease and infection was found to increase with the age of the plant.

At 10 days after first spray, the treatments with imidacloprid alone (T_1) or in combination with Perfekt / Action- 100 (T_7 and T_9) showed minimum incidence of 16.67 per cent followed by Perfekt alone (T_6) and *Pseudomonas fluorescens* + carbendazim - T_{12} (25 %). In addition, *Clerodendron* leaf extract + 1 % cow milk (T_4) and 2% neem oil spray (T_2) also provided protection to certain extent. *Gliricidia* leaf extract with cow milk was found to be least effective.

After second spray, the same treatments imidacloprid alone (T_1) and imidacloprid + Perfekt (T_7) recorded the minimum incidence of 27.77 and 29.27 per cent against 75 per cent in control followed by Perfekt alone (39.37%). A drastic increase in infection was noticed in the treatments, T_{12}, T_4 and T_2 which were found effective at the early stage of infection.

After third spray also, same trend was observed in which imidacloprid + Perfekt treatment showed lowest per cent incidence of 41.67 followed by imidacloprid alone (58.33%) and Perfekt alone (60.23%) against 83.30 per cent in control. All other treatments were on par with the control.

Table – 19 Effect of different treatments on disease incidence

Tr. No.	Treatments	Mean per cent disease incidence		
		After first spray	After second spray	After third spray
T_1	Imidacloprid (0.3ml/l)	16.67 [c] (4.055)	27.77[d] (5.304)	58.33[b] (7.634)
T_2	Neem oil – 20ml/l	28.47 [bc] (5.204)	72.20[ab] (8.472)	80.53[a] (8.99)
T_3	*Gliricidia maculata leaf extract* (10%) + 1% milk	47.23[ab] (6.836)	72.20[ab] (8.485)	80.53[a] (8.969)
T_4	*Clerodendron infortunatum* leaf extract (10%) + 1% milk	27.77[bc] (5.304)	62.77[abc] (7.916)	79.43[a] (8.938)
T_5	*Lawsonia inermis* (Henna) leaf powder (4g/l) + 1% milk	30.57[abc] (5.434)	53.23[abc] (7.209)	79.53[a] (8.935)
T_6	Perfekt – 2ml/l	25.00[bc] (5.004)	39.37[cd] (6.302)	60.23[b] (7.754)
T_7	Imidacloprid 0.3ml/l + Perfekt 2ml/l	16.67[c] (4.055)	29.27[d] (5.447)	41.67[c] (6.472)
T_8	Action 100- 2ml/l	33.37[abc] (5.789)	70.80[ab] (8.441)	78.17[a] (8.867)
T_9	Imidacloprid 0.3ml/l + Action 100- 2ml/l	16.67[c] (4.147)	45.80[bcd] (6.762)	79.17[a] (8.924)
T_{10}	*Pseudomonas fluorescens* - 20g/l	45.80[abc] (6.308)	58.30[abc] (7.545)	70.80[ab] (8.422)
T_{11}	*Pseudomonas fluorescens* 20g/l + Perfekt-2ml/l	41.70[ab] (6.496)	66.60[ab] (8.191)	66.70[ab] (8.198)
T_{12}	*Pseudomonas fluorescens* (10g/l) + carbendazim(1g/l)	25.00[bc] (5.004)	62.50[abc] (7.911)	70.80[ab] (8.422)
T_{13}	*Verticillium lecanii* -5g/l	30.57[bc] (5.321)	58.30[abc] (7.624)	72.20[ab] (8.514)
T_{14}	Curd- 10%	36.07[abc] (6.039)	71.18[ab] (8.451)	82.80[a] (9.127)
T_{15}	Lime (5g/l) + turmeric powder (10g/l)	37.50[abc] (6.158)	49.80[abc] (7.076)	79.43[a] (8.938)
T_{16}	Yellow sticky trap	36.13[abc] (6.014)	63.30[abc] (7.876)	80.53[a] (8.99)
T_{17}	Control	58.33[a] (7.657)	75.00[a] (8.689)	83.30[a] (9.154)

$\sqrt{x + 0.5}$ transformed values are given in parentheses

Figures followed by same letters do not differ significantly according to DMRT

Plate: 14 Effect of treatments on ToLCV infection

General view of the experimental plot

Imidacloprid + Perfekt treated plot

Control plot

4.6.2. Effect of different treatments on disease severity

All treatments were superior to control in reducing the disease severity. At 10 days after the first spray, treatments of imidacloprid with Action -100 (T_9) or Perfekt (T_7), *Pseudomonas fluorescens* + Perfekt (T_{11}) and imidacloprid alone (T_1) showed lowest severity of 4.4, 5.2, 6.3 and 6.9 per cent respectively against 21.16 per cent in control. Application of *Clerodendron* leaf extract + 1 % milk (T_4) also reduced the severity of the disease. *Gliricidia* leaf extract + 1 % cow milk (T_3) showed the maximum severity of 20.13 per cent. (Table – 20)

After the second spray also minimum severity was recorded with imidacloprid alone (16.3 %) followed by imidacloprid with Action -100 (18.3%) or Perfekt (20.83%) and Perfekt alone (23.33 %).

After the third spray minimum severity (26.03%) was observed with imidacloprid + Perfekt. Treatments with imidacloprid alone (32.06 %) and Perfekt alone (32.67%) were on par and the control plots recorded 67.3 per cent severity.

4.6.3. Effect of different treatments on coefficient of infection (CI)

It is noted from the Table –21 that, the minimum CI values were observed with imidacloprid + Perfekt- T_7 (10.78) followed by imidacloprid alone - T_1 (19.50) and Perfekt alone- T_6 (19.53) against 56.33 in control. It is also observed that, these treatments gave 80.84 to 65.3 per cent reduction over control. In addition *Pseudomonas fluorescens* +Perfekt could also reduce the infection to 50.71. It may also be emphasized that, in the present study, treatments with *Gliricidia* leaf extract, curd, yellow sticky trap and Action-100 alone did not give a satisfactory control of tomato leaf curl disease.

Table – 20 Effect of different treatments on disease severity

Tr. No.	Treatments	Mean per cent disease severity		
		After first spray	After second spray	After third spray
T_1	Imidacloprid (0.3ml/l)	6.967^{def} (2.555)	16.300^{f} (4.035)	32.067^{ef} (5.674)
T_2	Neem oil – 20ml/l	11.800^{abcdef} (3.496)	41.00^{abc} (6.382)	61.833^{abc} (7.876)
T_3	*Gliricidia maculata leaf extract* (10%) + 1% milk	20.133^{ab} (4.520)	43.00^{ab} (6.590)	64.467^{a} (8.059)
T_4	*Clerodendron infortunatum* leaf extract (10%) + 1% milk	7.633^{cdef} (2.847)	34.767^{abcde} (5.891)	59.867^{abc} (7.761)
T_5	*Lawsonia inermis* (Henna) leaf powder (4g/l) + 1% milk	10.333^{abcdef} (3.273)	35.667^{abcde} (5.927)	51.00^{abc} (7.137)
T_6	Perfekt – 2ml/l	10.033^{bcdef} (3.153)	23.300^{cdef} (4.789)	32.667^{def} (5.758)
T_7	Imidacloprid 0.3ml/l + Perfekt 2m/l	5.200^{ef} (2.381)	20.833^{def} (4.585)	26.033^{f} (5.150)
T_8	Action 100- 2ml/l	14.60^{abcd} (3.880)	40.967^{abc} (6.424)	65.300^{a} (8.112)
T_9	Imidacloprid 0.3ml/l + Action ·100- 2ml/l	4.400^{f} (2.213)	18.367^{ef} (4.337)	48.767^{abcde} (7.009)
T_{10}	*Pseudomonas fluorescens* - 20g/l	12.500^{abcdef} (3.352)	31.433^{bcdef} (6.314)	51.033^{abcd} (7.040)
T_{11}	*Pseudomonas fluorescens* 20g/l + Perfekt-2m/l	6.300^{cdef} (2.608)	27.100^{bcdef} (5.254)	41.633^{cde} (6.491)
T_{12}	*Pseudomonas fluorescens* (10g/l) + carbendazim(1g/l)	8.933^{cdef} (3.070)	28.500^{bcdef} (5.368)	42.833^{bcde} (6.542)
T_{13}	*Verticillium lecanii* -5g/l	9.067^{cdef} (2.974)	36.467^{abcd} (6.064)	52.083^{abc} (7.212)
T_{14}	Curd- 10%	13.933^{abcde} (3.754)	40.033^{abc} (6.314)	62.890^{ab} (7.940)
T_{15}	Lime (5g/l) + turmeric powder (10g/l)	11.467^{abcdef} (3.457)	38.800^{abcd} (6.252)	59.633^{abc} (7.753)
T_{16}	Yellow sticky trap	15.733^{abc} (4.010)	42.800^{ab} (6.546)	59.640^{abc} (7.727)
T_{17}	Control	21.167^{a} (4.568)	53.167^{a} (7.277)	67.633^{a} (8.223)

$\sqrt{x + 0.5}$ transformed values are given in parentheses

Figures followed by same letters do not differ significantly according to DMRT

Table – 21. Effect of different treatments on coefficient of infection

Tr. No.	Treatments	Mean values of coefficient of infection			Per cent reduction over control after third spray
		After first spray	After second spray	After third spray	
T_1	Imidacloprid (0.3ml/l)	1.509[cde] (1.318)	4.421[d] (2.196)	19.508[de] (4.383)	65.37
T_2	Neem oil – 20ml/l	3.544[bcde] (1.938)	31.145[a] (5.480)	50.124[ab] (7.085)	11.03
T_3	*Gliricidia maculata leaf extract* (10%) + 1% milk	9.663[ab] (3.130)	31.459[a] (5.604)	52.139[ab] (7.220)	7.45
T_4	*Clerodendron infortunatum* leaf extract (10%) + 1% milk	2.138[cde] (1.617)	22.969[ab] (4.724)	47.370[abc] (6.916)	15.91
T_5	*Lawsonia inermis* (Henna) leaf powder (4g/l) + 1% milk	3.487[bcde] (1.914)	21.471[abc] (4.418)	41.103[abc] (6.387)	27.04
T_6	Perfekt – 2ml/l	2.832[cde] (1.745)	9.608[bcd] (3.089)	19.539[de] (4.461)	65.32
T_7	Imidacloprid 0.3ml/l + Perfekt 2ml/l	0.992[de] (1.174)	6.270[cd] (2.566)	10.789[e] (3.353)	80.84
T_8	Action 100- 2ml/l	4.755[abcde] (2.290)	29.198[a] (5.427)	51.058[ab] (7.178)	9.37
T_9	Imidacloprid 0.3ml/l + Action 100- 2ml/l	0.735[e] (1.111)	8.616[bcd] (2.983)	38.763[abc] (6.250)	31.20
T_{10}	*Pseudomonas fluorescens* - 20g/l	8.325[abc] (2.580)	21.801[abc] (4.327)	38.127[abcd] (6.008)	32.33
T_{11}	*Pseudomonas fluorescens* 20g/l + Perfekt-2m/l	2.627[cde] (1.768)	18.049[abc] (4.307)	27.769[cd] (5.317)	50.71
T_{12}	*Pseudomonas fluorescens* (10g/l) + carbendazim(1g/l)	2.197[cde] (1.636)	18.279[abc] (4.281)	31.297[bcd] (5.549)	44.45
T_{13}	*Verticillium lecanii* -5g/l	3.830[bcde] (1.847)	21.853[abc] (4.662)	38.179[abc] (6.154)	32.23
T_{14}	Curd- 10%	5.160[abcde] (2.339)	29.337[a] (5.369)	52.126[ab] (7.233)	7.48
T_{15}	Lime (5g/l) + turmeric powder (10g/l)	4.329[bcde] (2.192)	19.712[abc] (4.453)	47.321[abc] (6.914)	16.01
T_{16}	Yellow sticky trap	5.865[abcd] (2.484)	28.986[a] (5.246)	52.572[ab] (7.217)	6.69
T_{17}	Control	12.901[a] (3.556)	39.875[a] (6.312)	56.339[a] (7.511)	

$\sqrt{x + 0.5}$ transformed values are given in parentheses

Figures followed by same letters do not differ significantly according to DMRT

4.6.4. Effect of treatments on biometric characters of the plant

The biometric characters such as plant height, days to flowering, days to fruiting, days to harvest, number of fruits per plant, yield per plant, yield per plot were recorded and presented in Table – 22. With respect to the biometric characters any noticeable difference could be observed among the treatments except in case of plant height, yield / plants and yield / plot, in which maximum height (71 cm) was noted in treatment with *Pseudomonas fluorescens* + carbendazim (T_{12}) and maximum yield per plant were recorded in T_7 (imidacloprid + Perfekt) followed by T_1 (imidacloprid alone).

In case of yield per plot, imidacloprid + Perfekt (T_7) was significantly superior to other treatments recording the highest yield of 3.22 kg followed by imidacloprid alone (T_1) with 2.82 kg and *Pseudomonas fluorescens* + Perfekt (T_{11}) with 2.17 kg and the lowest yield (0.36 kg) was recorded in control plot. It is also noted that these treatments were also effective in reducing the ToLCV incidence.

Summing up the above findings, it is observed that, the insecticide imidacloprid in combination with botanical Perfekt/ Action-100 or imidacloprid alone or Perfekt alone were the best treatments for the management of ToLCV. In addition, application of *Pseudomonas fluorescens* + Perfekt also recorded good yield and reduced the infection to certain extent.

Table – 22 Effect of different treatments on biometric characters of the plant

Tr. No.	Treatments	Plant height (cm)	Days to flowering DAP	Days to fruiting DAP	Days to first harvest DAP	Average number of fruit / plant	Average weight of fruits (g)	Yield/plant (g)	Yield/plot (Kg/3.24m^2)
T_1	Imidacloprid (0.3ml/l)	64.67	24	34	54	9.3	26.2	250.00	2.82^{b}
T_2	Neem oil – 20ml/l	57.33	26	37	57	2.6	24.5	56.90	0.49^{gh}
T_3	*Gliricidia maculata leaf extract* (10%) + 1% milk	50.00	30	44	66	2.5	22.4	57.90	0.46^{gh}
T_4	*Clerodendron infortunatum* leaf extract (10%) + 1% milk	47.67	26	39	58	3.1	21.1	76.00	0.67^{fgh}
T_5	*Lawsonia inermis* (Henna) leaf powder (4g/l) + 1% milk	52.00	25	38	57	5.1	20.0	130.00	1.12^{de}
T_6	Perfekt – 2ml/l	63.67	22	33	55	4.2	21.3	109.00	1.15^{de}
T_7	Imidacloprid 0.3ml/l + .Perfekt 2m/l	62.67	23	34	54	12.8	25.2	320.00	3.22^{a}
T_8	Action 100- 2ml/l	60.33	26	37	56	5.2	31.0	175.00	1.41^{d}
T_9	Imidacloprid 0.3ml/l + Action 100- 2ml/l	59.33	25	37	56	7.1	27.1	162.00	1.40^{d}
T_{10}	*Pseudomonas fluorescens* 20g/l	58.00	25	35	55	4.2	26.2	106.00	1.00^{def}
T_{11}	*Pseudomonas fluorescens* 20g/l + Perfekt-2m/l	63.00	24	37	54	5.6	29.2	200.00	2.17^{c}
T_{12}	*Pseudomonas fluorescens* (10g/l) + carbendazim(1g/l)	71.00	31	41	58	4.4	25.8	145.00	1.32^{d}

T_{13}	*Verticillium lecanii* -5g/l	54.00	29	41	56	5.1	28.2	138.00	1.24^{d}
T_{14}	Curd- 10%	57.33	21	35	57	1.9	25.0	54.00	0.47^{gh}
T_{15}	Lime (5g/l) + turmeric powder (10g/l)	60.67	25	38	56	2.7	26.1	70.00	0.64^{fgh}
T_{16}	Yellow sticky trap	55.67	27	38	55	3.2	24.8	90.00	0.81^{efg}
T_{17}	Control	51.20	27	38	57	1.8	25.2	46.00	0.36^{h}

Figures followed by same letters do not differ significantly according to DMRT

DAP- Days After Planting

Discussion

5. DISCUSSION

Tomato leaf curl virus (ToLCV) disease is one of the most important diseases of tomato which hamper its cultivation. The occurrence of tomato leaf curl disease was first reported in India by Pruthi and Samuel (1939) and subsequently by Vasudeva and Samraj (1948). The disease exists since several decades, threatening the commercial cultivation of tomato particularly during summer.

Recently, tomato leaf curl has become a serious problem in Kerala. Kerala Agricultural University has initiated some work on ToLCV, giving emphasis to breeding and molecular mapping. However, the information on existence of virus variants, molecular characterization of the virus and disease management aspects are lacking. Search on literature revealed that, much attention has not been received on this disease in Kerala.

Keeping all these in mind, an investigation was undertaken to study the symptomatology, molecular characterisation of virus and disease management strategies, which would add to our knowledge especially on the existence of variants of the causal agent.

The survey was conducted to assess the incidence, severity and to record the symptoms ToLCV in major tomato growing areas of Palakkad *viz.* Kozhinjampara, Vandithavalam, Perumatty, Vadakarapathy and Eruthempathy and also in Vellanikkara campus during rabi and summer seasons. The results revealed that, the per cent disease incidence varied from location to location and season to season.

During rabi season, the incidence was comparatively less in both Palakkad and Vellanikkara, recording 2 – 6 and 22 – 47 per cent respectively. But the disease incidence was higher during summer, which ranged from 74 to cent per cent in Palakkad and 64 – 88.8 per cent in Vellanikkara. The disease severity was also higher in both Palakkad and Vellanikkara, during summer months. Same

trend was recorded by Reddy (1978) with 6– 38 per cent incidence in rabi and 25–86 per cent in summer. Similar observations were also made by Saikia and Muniyappa (1989), Raghavendra (2002), Reddy *et al.* (2011) from Karnataka areas and Saha *et al.* (2014) from West Bengal.

The meteorological data recorded during the survey period showed low temperature, high humidity and high rainfall during rabi. The high rainfall during rabi season which was unfavourable for the buildup of whitefly population and it might have contributed for less disease incidence. In summer, the mean temperature was found to be 27.95 to 30.45^{0}C with less relative humidity of 51–56 percent and there was no rain fall, which favoured the whitefly population and might be the reason for the high incidence of tomato leaf curl disease during summer months. Tripathi and Varma (2002) reported that, minimum temperature and minimum relative humidity influenced the whitefly population in north India.

Reddy (2006) also noted that, whitefly population was positively correlated with maximum and minimum temperature and negatively correlated with relative humidity. This gives a very clear picture that, low humidity and high temperature is congenial for the activity of the whitefly, in turn for the rapid spread of ToLCV disease. Similar results of seasonal variations of whitefly population have been observed by Pruthi and Samuel (1939); Reddy (1978); Makkouk *et al.*, 1979; Saikia (1985).

During the survey, four types of symptoms were found associated with ToLCV in Vellanikkara, such as, upward curling and cupping (Type I), curling and rolling (Type II), curling with purple tint (Type III) and yellowing and curling (Type IV). Whereas in Palakkad areas, upward curling and cupping and curling and rolling were more prominent as compared to rolling with purple tint. Yellowing and curling type were not observed in these areas. All the varieties/ hybrids grown in the surveyed areas were found susceptible to this disease.

Symptoms are the observable effects that a pathogen causes on the growth, development and metabolism of an infected host plant. Study on symptomatology is an important criterion for the early detection and identification of crop diseases. The characteristic symptoms of the disease were curling and puckering of the leaves, stunted growth, reduction in leaf size and internodal length resulted in bushy appearance The flowers were completely or partially sterile. The infected plants produced few fruits and no fruits depend on the stage of crop at infection occurs. Same types of symptoms were described by various other workers with slight variations (Vasudeva and Samraj, 1948; Nariani, 1968; Sastry and Sigh, 1973; Saklani and Mathai, 1977; Saikia and Muniyappa, 1989; Reddy, 2006; Saha *et al.*, 2014).

In the present study, four different types of symptoms were observed on the infected plants under natural conditions *viz.* upward curling and cupping, curling and rolling, curling with purple tint and yellowing and curling. Similar types of symptoms were also recorded by Nazeem *et al.* (2006) and Divakaran (2007). However, these groups are different from those codified by Muniyappa (1994). The appearances of purple patches, especially on older leaves were noted by other workers also (Vasudeva and Samraj, 1948; Saklani and Mathai, 1977; Saikia and Muniyappa, 1989). Yellowing and curling type symptom noticed in Kerala is not observed elsewhere in India. However an yellow leaf curl virus disease of tomato has been reported from other countries. The enation on the lower surface of the leaves reported by Nariani (1968) was not observed in the present study. Variation in the symptoms may be attributed due to the existence of different virus strains/mutants (Padidam *et al.*, 1995).

All the four different types of symptoms observed under the natural conditions were also noticed on artificial inoculation. Flowering was not observed in any of the artificially inoculated plants. Furthermore, the severity of the symptoms was also high due to early infection.

Transmission is an important criterion to establish the etiology of viral diseases. Graft and whitefly transmission studies were conducted with the infected plants showing all four types of symptoms. Since the viruses are systemically distributed in plants, it can be transmitted through grafting. Vascular connection between stock and scion facilitate movement of viral particles and plays a significant role in the transmission of viruses. Grafting is not an important or common mean of natural spread of viruses and is often used as an experimental tool to take decisive evidence of viral and mycoplasmal etiology of the disease. Among the two methods of grafting, approach grafting showed 50 – 70 per cent transmission and early symptoms with 14 – 17 days of incubation period, whereas in wedge grafting, it was 10 – 30 per cent and 24 – 28 days respectively. Thus, these findings showed that, approach grafting is the better method for the transmission studies. Tanaka (1962) also observed that, approach grafting is suitable for the mechanical transmission of viruses. However due to the difficulty in getting large number of infected plants matched with the same stem size of root stock, wedge grafting was adopted as common method by various workers. Both methods showed successful transmission by producing the four types of symptoms on the grafted plants.

The vector mediated transmission is a well known technique in plant virology. The earlier workers extensively studied the transmission of ToLCV by whitefly, *Bemisia tabaci* and the virus vector relationship (Vasudeva and Samraj, 1948; Ramakrishnan *et al.*, 1964; Saikia, 1985; Muniyappa *et al.*, 2000; Naik, 2001; Rajasri *et al.*, 2011; Saha *et al.*, 2014).

In view of these earlier successes it seemed logical to attempt the transmission of ToLCV using whitefly, *B. tabci.* The transmission studies were conducted using all the four different types of symptoms and the same type of symptoms could be reproduced on the test plants through whiteflies. The transmission studies conducted during January- March (summer) months yielded high rate of transmission of 40-90 per cent as compared to that in October – December (45–60 per cent).

The incubation period was also found to be less in summer, showing 16–18 days against 27–33 days in rabi. The transmission rate and incubation period were found to vary with the prevailing temperature. Kunkel (1948) reported a correlation between the length of incubation period in the host plant and leaf hopper vectors at optimal temperature, both in aster yellows and corn stunt diseases. He found that, the incubation period of corn stunt pathogen was shorter during autumn than winter days in glasshouse condition. Kassanis (1957) also reported the direct effect of temperature on the disease development process in virus infected plants. Mathew (1983) observed shorter incubation period of grassy stunt virus in rice plant during summer (14– 21 days) as compared to that in winter (65– 72 days). This showing that, temperature plays an important role in virus infection.

Reading back the results of the survey conducted at Palakkad and Vellanikkara in which the disease was found to be severe in summer months indicating that, the high temperature may be a factor favoring the ToLCV infection.

The next point to be discussed is the serodiagnosis of ToLCV. Serological relationship of ToLCV with other geminiviruses was established using ELISA which is a very sensitive test for detection and identification of virus. In the present study, ToLCV antigen was tested against one polyclonal and two monoclonal antibodies of geminivirus. The positive reaction of ToLCV was obtained only with the polyclonal antiserum. Swanson *et al.* (1992) reported that, as there is 60 per cent conservation in the coat protein gene that polyclonal antibodies raised against any *Begomovirus* can react with all the begomoviruses.

DAC-ELISA employed to detect the presence of ToLCV in the samples of four different types of symptoms, showed positive reaction with the polyclonal antibody of *African cassava mosaic virus* (ACMV) and confirmed the presence of gemini virus. However, the value of absorbance varied with samples (0.7625–

1.0095) and may be due the variation in concentration of virus in the infected samples.

The virus associated with all four types of symptoms of ToLCV is found serologically related to *African cassava mosiac virus*. This is in agreement with the findings of Muniyappa *et al.* (1991b), Swanson *et al.* (1992), Muniyappa (1994), and Reddy (2006) who also reported the relationship of ToLCV with ACMV. ToLCV isolate from Australia is also serologically related to ACMV as per ICTVdB- the universal virus database (2006).

All the four ToLCV infected samples showed negative reactions with the monoclonal antibodies of *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) indicating that this virus is not related to ICMV and SLCMV strains. This is in line with the findings of Muniyappa *et al.* (1991b) who also stated that, monoclonal antibodies raised against ICMV could not be used to differentiate ToLCV. Zacharia (2006) reported that, the begomovirus associated with *Bitter gourd distortion mosaic virus* in Kerala was found serologically similar to tomato yellow leaf curl virus.

The above serological results proved that, the causal agent of four different types of symptoms associated with tomato leaf curl disease in Kerala is a virus belonging to genus *Begomovirus* of family *Geminiviridae*.

On recalling the observations in the symptomatology and the appearance of different types of symptoms in the transmission studies, strengthened the possibility of existence of different strains or variants of ToLCV in Kerala. Hence an attempt was made to find out the existence of different variants of ToLCV in Kerala through molecular approaches.

Molecular approach has gained more importance recently in the detection and characterization of plant pathogens.The polymerase chain reaction (PCR) provides a simple, ingenious method to exponentially amplify specific DNA sequences by *in vitro* DNA synthesis. PCR techniques overcome the problems

associated in serological detection methods and has been utilized successfully to detect geminiviruses (Rojas *et al.*, 1993 and Deng *et al.*, 1994).

Isolation of good quality DNA is prerequisite for performing PCR. The total plant DNA from ToLCV infected samples showing four different types of symptoms were isolated by the method described by Rogers and Bendich (1994). Certain modifications like increasing the spinning rate and alteration in the incubation time were adopted to yield good quality DNA. This method was found suitable for the extraction of ToLCV infected plants. Divakaran (2007) also reported that, protocol suggested by Rogers and Bendich (1994) with modifications was found to be most appropriate for DNA isolation from tomato leaves. The reaction of optical density value at 260nm to that at 280nm ranged from 1.732 – 1.90. The value of OD_{260}/OD_{280} between 1.8 and 2 indicates relatively pure DNA.

Attempts were also made to standardize the molecular detection of ToLCV with already reported primers. The primers reported by Reddy *et al.* (2005) (CRv 301 and CRc1152) and Redyy *et al.* (2011) (ToLCBV33F and ToLCBV 1070R) were used to amplify the coat protein of ToLCV from the samples showing four different types of symptoms. Initially, optimization of the PCR conditions were carried out since the PCR parameters are very critical to amplify the DNA fragments with specific primers. The tested parameters were annealing temperature and template concentration. In the present study, for the primers CRv 301 and CRc1152, the annealing temperature ranging 47– 53^0C was tried and optimum amplification of product was obtained at 51^0C.

Template concentration was another parameter that was standardized. Optimum amplification was obtained with a template concentration of 37 ng/µl. With a decrease in template concentration a decrease in intensity of amplification was observed.

PCR amplifications were carried out with the primers CRv 301 and CRc1152 (Reddy *et al.*, 2005) and the amplified product of expected size of 870

was observed with four different samples under investigation. Thus it was confirmed that the four different types of symptoms observed in Kerala are associated with tomato leaf curl disease. Thus these findings support the report of Reddy *et al.* (2005) and Saha *et al.* (2014) who also detected ToLCV with the same set of primers.However, no amplification was obtained with the primer set ToLCBV33F and ToLCBV 1070R in different set of annealing temperature and template dilution. This is in contradiction to the findings of Reddy *et al.* (2011) who obtained an amplified 1040 bp product with the primers, ToLCBV33F and ToLCBV 1070R.

ToLCV coat proteins from plants exhibiting four different types of symptoms were sequenced and the phylogenetic analysis showed that, the four sequences vary from one another. The upward curling and cupping type and curling and rolling type were in same clade whereas curling with purple tint and yellowing and curling type formed another clade in the phylogenetic tree. Hence, the variation in symptoms can be attributed to the difference in nucleotide sequence. Boulton *et al.* (1991) also reported that, variation in a few nucleotides can cause major phenotypic differences. Pandey *et al.* (2010) showed that, two ToLCV isolates from Kerala ToLCK3 and ToLCK5 are distinct from each other.

Several species and strains of begomovirus causing ToLCD have been reported (Fauquet *et al.*, 2003). In Indian subcontinent, 12 species are known to be associated with the disease. They are, *Tomato leaf curl New Delhi virus* (ToLCNDV) (Padidiam *et al.*, 1995), *Tomato leaf curl Bangalore virus* (ToLCBV) (Muniyappa *et al.*, 2000), *Tomato leaf curl Karnataka virus* (ToLCKV) (Chatchawankaphanich and Maxwell, 2002), *Tomato leaf curl Gujarat virus* (ToLCGV) (Chakraborty *et al.*, 2003), *Tomato leaf curl Bangladesh virus* (ToLCBDV), *Tomato leaf curl Sri Lanka virus* (ToLCVSLV), (Rishi, 2004), *Tomato leaf curl Joydebpur virus* (ToLCJV) (Maruthi *et al.*, 2006), *Tomato leaf curl Palampur virus* (ToLCPMV) (Kumar *et al*, 2008), *Tomato leaf curl Kerala*

virus (ToLCKeV) (Pandey *et al.*, 2010), *Tomato leaf curl Patna virus* (ToLCPaV) (Kumari *et al.*, 2010), *Tomato leaf curl Pune virus* (ToLCPuV) and *Tomato leaf curl Rajasthan virus* (ToLCRV) (Varma *et al.*, 2011).

Begomoviruses have circular single stranded DNA bipartite genome components, DNA-A and DNA-B. The existence of DNA β, a single-stranded circular satellite DNA was reported in some members of the begomogenus (Saunders *et al.*, 2000; Briddon *et al.*, 2001 and Zhou *et al.*, 2003). The most significant variation in the genomes of begomoviruses is the lack of DNA-B in monopartite begomoviruses. DNA-A is associated with the replication of the virus proteins, DNA-B codes for the virus movement and DNA β is found to be associated with the severity of the symptom. *Tomato leaf curl Patna virus* is reported to posses DNA β.

However, ToLCNDV, ToLCGV and ToLCPV reported from northern India are bipartite that require both DNA-A and DNA-B for causing infection in the host plant. Chakraborty *et al.* (2003) have cloned, sequenced DNA-A and DNA-B of components of two isolates from Varanasi and Mirzapur and identified as similar to ToLCGV. Whereas, the south Indian isolates are reported to be monopartite with only DNA-A component (Muniyappa *et al.*, 2000).

The four sequences obtained from four different types of symptoms were compared using bioinformatics tools. The blast hits showed that all the four sequences are having maximum homology with the DNA-A component of other monopartite begomoviruses. This is in accordance with the findings obtained by Muniyappa *et al.* (2000) and Reddy *et al.* (2005) who observed only DNA –A in south Indian isolates. Pandey *et al.* (2010) also, could not detect DNA–B and β in the two Kerala isolates. The findings in the present investigation showed that, the four sequences of ToLCV infecting tomato in Kerala are similar to monopartite begomoviruses with respect to the coat protein region. However, to confirm this, complete genome sequencing is imperative.

For the better understanding of the variation of the four ToLCV isolates under investigation, it is essential to discuss the data of blast hits and phylogenetic analysis. The sequences showed significant homology with different monopartite begomoviruses present in NCBI database.

Analysis of similarity search of upward curling and cupping type ToLCV isolate exhibited maximum identity (96 per cent) to *Ageratum yellow vein Sri Lanka virus* (AF3314144.1) and *Tomato leaf curl virus* isolated from *Ageratum* (AJ810367.1). However, in the phylogenetic tree, upward curling and cupping type ToLCV isolate was grouped in the same clade of tomato leaf curl Karnataka isolates (Fig. 2). This is similar to the observations made by Reddy *et al.* (2005), the sequences obtained by them also grouped with other Karnataka isolates in the phylogenetic analysis.. In the host range studies conducted by Sastry (1984), Saikia and Muniyappa (1989); Naik (2001) and Reddy (2006) *Ageratum conyzoides* was found to be a collateral host of ToLCV.

The sequence of curling and rolling type was found to be more similar to the chilli leaf curl virus with 96 per cent identity with the DNA- A segment. This is line with the findings of Reddy *et al.* (2005) who observed similarity with chilli leaf curl virus while cloning and sequencing of tomato samples with ToLCV symptoms from Calcutta. Pandey *et al.* (2010) also observed 95 per cent similarity of coat protein gene (AV1) of the two Kerala isolates with chilli leaf curl virus. Thus the present findings confirmed their results. Morilla *et al.* (2005) and Polston *et al.* (2006) observed the infection of TYLCV on chilli and demonstrated the ability of chilli plants to serve as symptomless reservoir.

Yellowing and curling type and curling with purple tint type sequences were also compared and blasted. Both types were found to be 82-83 per cent identical with the three ToLCV Kerala sequences available in the NCBI database. These sequences showed similarity to the ToLCV K3 and ToLCV K5 Kerala isolates reported by Pandey *et al.* (2010). Hence these two types of symptoms in the present study are confirmed to be ToLCV. However, there is variation in the

nucleotide sequences with respect to the coat protein region. Further demarcation of these isolates is possible only with the complete genome sequencing of the isolates as suggested by Fauquet *et al.* (2008).

The present findings revealed that, of the four types of symptoms observed in Kerala, two types of symptoms yellowing and curling type and curling with purple tint are identical with the already reported ToLCV K3 and ToLCK5 strains, however upward curling and cupping type and curling and rolling types are different variants which showed similarity to *Ageratum yellow vein mosaic virus* and *Chilli leaf curl virus*. From these findings, it is observed that, the virus causing ToLCD in Kerala is one of the strains similar to the Kerala strain reported by Pandey *et al* (2010). No previous report is available which states that, the ToLCD inciting viruses from Kerala are showing homology to *Ageratum yellow vein mosaic virus*.

The diversity of begomoviruses in tomato in Kerala determined in this study represents general trends as coat protein sequence represent the most conserved region of the begomovirus genome (Wyatt and Brown, 1996; Fauquet, 2008). The four sequences obtained in this study showed homology to the monopartite begomoviruses genome only. None of them had shown similarity to the bipartite begomoviruses reported from North India or elsewhere. Furthermore, Pandey *et al.* (2010) could detect only DNA-A in the two Kerala isolates of ToLCV with complete genome sequencing. Muniyappa *et al.* (2000) and Reddy *et al.* (2005) also found that south Indian isolates of ToLCV are monopartite with DNA–A component which are supporting the findings of the present investigation.

Open reading frame is the part of protein coding gene and the longest ORF of the DNA sequence is considered to detect the protein/polypeptide coded. It starts with an initiation codon and ends with a termination codon (Old and Primrose, 1994). The ORFs of all the four isolates were examined and when subjected BLASTp search, detected homology with coat protein of geminiviruses

only. This data further confirmed the molecular basis of ToLCV infection with the four different symptoms detected in Kerala.

It is known that, CP genes in the begomoviruses are the most conserved ones (Brown *et al.*, 2001). Therefore, differences in CP gene sequence could not detect begomovirus with in the species level. Because of the growing number of species under this virus, derivation of the complete nucleotide sequence of the virus has become necessary to distinguish the species (Kings *et al.*, 2011). However, in the present study sequence analysis of CP gene provided valuable information about the cause of tomato leaf curl disease of Kerala that the four different types of symptoms associated with ToLCD is caused by begomovirus. But it is essential to sequence the complete genome of the viruses to ascertain the accurate species of begomoviruses causing this disease in Kerala. Partial genome sequencing indicating the existence of two more variants in Kerala in addition to ToLCV K3 and ToLCVK5 already reported by Pandey *et al.* (2010). However, this can be confirmed only by complete genome sequencing for which, Rolling Circling Amplification kit is highly essential which is quite expensive that, it is beyond the scope of present study.

An appropriate method for better management of disease in an integrated approach by the use of resistant varieties supplemented with cultural, chemical and biological methods. Breeding works are in progress to develop tomato varieties resistant to ToLCV. The management of disease by plant protection measures is found to be another alternative method.

Among the various methods, chemicals offer comparatively better management of plant viruses. However, the continuous use of chemicals may lead to the development of resistant biotypes of vectors, phytotoxicity and environmental pollution. Studies conducted on the use of botanicals and bioagents have opened a new avenue for the management of plant viral diseases. Recent emphasis on the development of non-chemical control methods has given impetus to more intensive explorations. Besides being safe and non-phytotoxic, certain

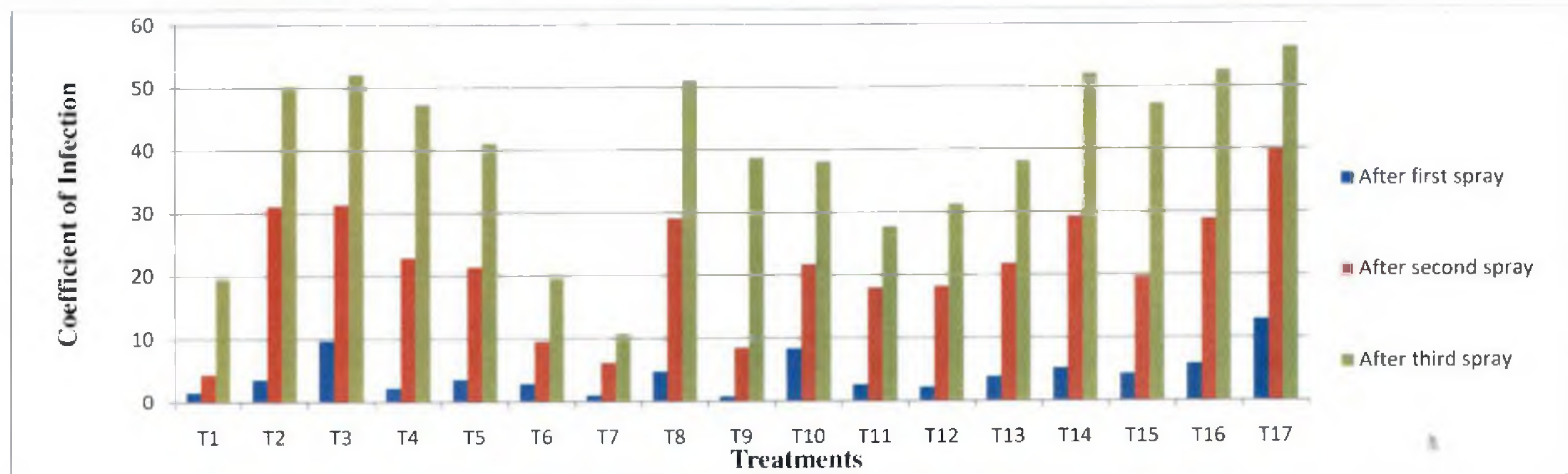
selected botanicals are known to be effective against various viral diseases and insect vectors. Many workers have observed the effect of botanicals in the suppression of viral diseases (Louis and Balakrishnan, 1996; Tewari *et al.*, 2001; Reddy, 2006; Al-ani *et al.*, 2011). Yellow sticky trap, a physical means for the control of whitefly population was reported by Krishnareddy (1989).

Recent studies about the efficacy of *Pseudomonas fluorescens* in suppressing the viral infection showed the ability for inducing systemic resistance in tomato plants. Keeping these aspects in mind, an attempt was made to develop management strategy with certain chemicals, botanicals and bioagents. These were evaluated to know their efficacy to suppress the disease or by keeping incidence at low level by controlling the vector, *B. tabaci,* which is responsible for the spread of the disease.

From the field experiment conducted, it was found that none of the treatments could give complete protection to ToLCV. However, the treatments were superior to control with respect to disease incidence, severity, coefficient of infection (Fig.6) and yield (Fig.7) and the effectiveness varied with treatments as well as the stage of infection. In the disease management studies, treatments with botanicals, insecticides, bioagents, and yellow sticky trap provided better management at early stages and the effect is found decreasing as the disease progressed.

In the current investigation, the application of commercial botanical, Perfekt and insecticide, imidacloprid alone or in combination could reduce the ToLCV infection and increase the yield. However, the combination spray of imidacloprid + Perfekt was found to be more effective. In addition, the application of imidacloprid with another botanical, Action-100 also showed reduction in ToLCV incidence and severity. This confirmed the findings of Bajapai (2005) who also reported the effectiveness of combined application of insecticide, imidacloprid with commercial botanical, Action -100 and Pre-Vental B.V. in managing the chilli leaf curl disease. Similar to this, Divya (2011) also reported

Fig.6. Effect of different treatments on coefficient of infection of ToLCV



T_1 -Imidacloprid (0.3ml/l)

T_2 -Neem oil – 20ml/l

T_3 -*Gliricidia maculata leaf extract* (10%) + 1% milk

T_4 -*Clerodendron infortunatum* leaf extract (10%) + 1% milk

T_5 -*Lawsonia inermis* (Henna) leaf (4g/l) + 1% milk

T_6 -Perfekt – 2ml/l

T_7 -Imidacloprid 0.3ml/l + Perfekt 2m/l

T_8 -Action 100- 2ml/l

T_9 -Imidacloprid 0.3ml/l + Action 100- 2ml/l

T_{10} -*Pseudomonas fluorescens* - 20g/l

T_{11} -*Pseudomonas fluorescens* 20g/l + Perfekt-2m/l

T_{12} -*Pseudomonas fluorescens* (10g/l) + carbendazim (1g/l)

T_{13} -*Verticillium lecanii* -5g/l

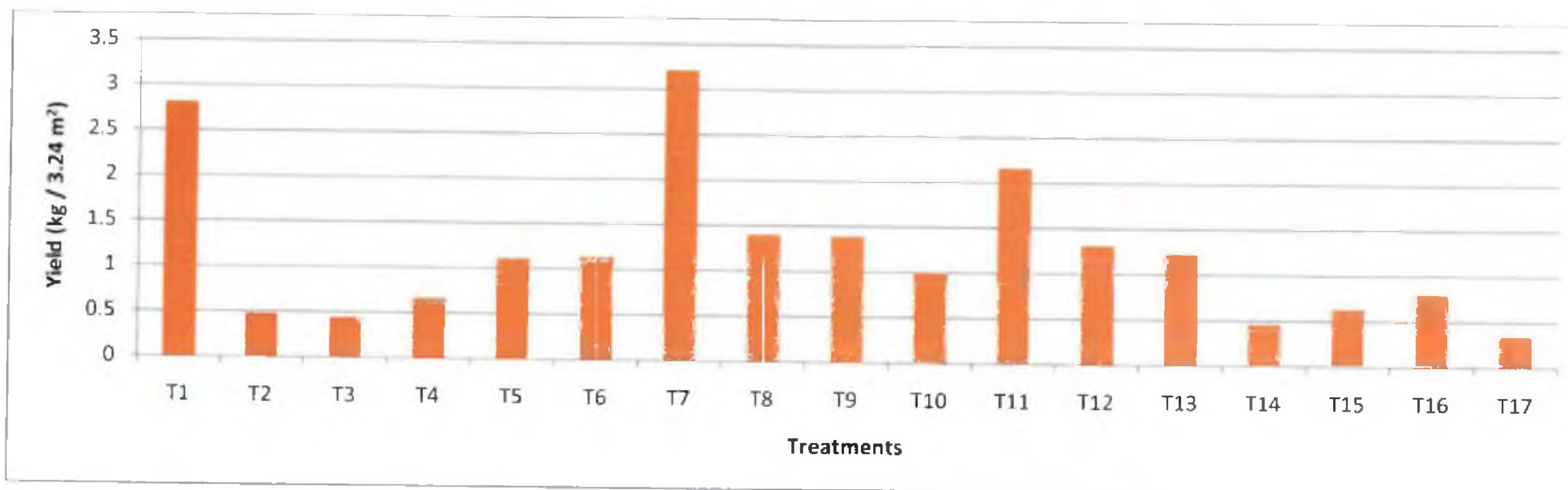
T_{14} -Curd- 10%

T_{15} -Lime (5g/l) + turmeric powder (10g/l)

T_{16} -Yellow sticky trap

T_{17} Control

Fig.7. Effect of different treatments on yield of tomato



T_1 -Imidacloprid (0.3ml/l)

T_2 -Neem oil – 20ml/l

T_3 -*Gliricidia maculata leaf extract* (10%) + 1% milk

T_4 *Clerodendron infortunatum* leaf extract (10%) + 1% milk

T_5 -*Lawsonia inermis* (Henna) leaf (4g/l) + 1% milk

T_6 Perfekt – 2ml/l

T_7 Imidacloprid 0.3ml/l + Perfekt 2m/l

T_8 -Action 100- 2ml/l

T_9 -Imidacloprid 0.3ml/l + Action 100- 2ml/l

T_{10} -*Pseudomonas fluorescens* - 20g/l

T_{11} -*Pseudomonas fluorescens* 20g/l + Perfekt-2m/l

T_{12} -*Pseudomonas fluorescens* (10g/l) + carbendazim (1g/l)

T_{13} -*Verticillium lecanii* -5g/l

T_{14} -Curd- 10%

T_{15} -Lime (5g/l) + turmeric powder (10g/l)

T_{16} -Yellow sticky trap

T_{17} Control

the effectiveness of quinalphos + Action- 100 for the management of ash gourd mosaic disease. Reddy (2006) reported that application of 0.05 per cent imidacloprid at 15 days interval could reduce the ToLCD incidence to 28.33 per cent at 90 days after transplanting. Bajapai (2005) and Zacharia (2006) also obtained reduction in chilli leaf curl and bittergourd distortion mosaic diseases respectively with spray of 0.025 per cent imidacloprid, thus supporting the present findings.

In the present study, it was found that, application of *Clerodendron infortunatum* leaf extract, henna leaf powder, *Gliricidia maculata* leaf extract and neem oil 2%, performed better in reducing the disease incidence at early stage. The plant products are reported to have antiviral properties which may act directly by inactivating the virus multiplication or indirectly by increasing the host resistance and also have repellant action against the vector resulting in reduction of virus infection. However, these treatments did not provide better management as the disease progressed. This is in agreement with the findings of Reddy (2006) and Al-ani *et al.* (2011) who also observed that *Clerodendron sp, Gliricidia sp* and henna extract could reduce the incidence of tomato leaf curl disease only at early stage of the crop. Similar observation has been recorded by Zacharia (2006) with respect to the neem oil 2% in the management of whitefly transmitted bitter gourd distortion mosaic.

It is interesting to note that, eventhough the locally prepared leaf extracts did not show much effectiveness against ToLCV, the commercial botanicals, Perfekt and Action-100 could provide protection against ToLCV to certain extent and among these two, Perfekt was found to be more effective. Arunakumara *et al.* (2010) and Meena (2012) have reported the efficacy of Perfekt in reducing the incidence of early blight of tomato and purple blotch of onion respectively. Search on literature revealed no information on the effect of Perfekt in the management of any viral disease and perhaps this may be the first attempt on a virus disease.

In addition, application of *Pseudomonas fluorescens* also showed reduction in ToLCV infection and better performance with Perfekt botanical. Samiyappan (2003) suggested the efficacy of *P. fluorescens* in reducing the virus infection in tomato and Mishra *et al.* (2014) also noted the reduction in ToLCV with chitosan supplemented formulations of *P. fluorescens* which may be due to induced systemic resistance.

It is also noted that, *P. fluorescens* + carbendazim treatment was also effective in the initial stages. The effectiveness of carbendazim fungicide in the suppression of TMV and lettuce western yellows disease has been already reported by Tomilson *et al.* (1976). Thomas and John (1980) also observed the suppression of tungro virus disease of rice plants with application of carbendazim. Moreover, the plant height was also found to be maximum in the *P. fluorescens* + carbendazim treated plants and this may be due to the stimulating effect of carbendazim. Nene and Tapalial (1971) reported the growth promoting activity of carbendazim.

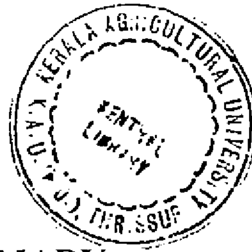
The antiviral property of milk and casein products is well known facts. Averre and Gooding (2000), Lewandowski *et al.* (2010) and Abdelbacki *et al.* (2010) reported the effect of milk/casein products in reducing the viral infection in plants. Likewise, Krishnareddy (1989) obtained control of whitefly population with yellow sticky trap. However, in the present study neither the application of curd nor the use of yellow sticky trap gave a satisfactory control of tomato leaf curl disease.

Any treatment applied in disease management will be effective when it reduces the infection and at the same time give maximum yield. During the present investigation, maximum yield was also recorded in imidacloprid + Perfekt treated plots, followed by imidacloprid alone and *P. fluoresens* + Perfekt, which also gave better performance in disease suppression. The stimulating effect of fluorescent pseudomonads in growth and yield of tomato has been reported by other researchers (Samiyappan, 2003 and Gravel *et al.*, 2007).

Considering the overall performances of various treatments, it was found that treatment of imidacloprid in combination with the botanical, Perfekt or imidacloprid / Perfekt alone and *P. fluorescens* + Perfekt were the best treatments for management of tomato leaf curl disease. This may be well explained from the fact that increase in yield is obtained corresponding to decrease in infection.

Summing up the discussion so far, it may be concluded that the present studies have provided much information about the various aspects of ToLCV disease in Kerala, particularly symptomatology, existence of different variants, molecular characterisation and the use of commercial botanical, Perfekt in the management of virus disease.

Summary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6. SUMMARY

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown intensively in tropics and subtropics. The damage caused by diseases is the major constraint for the tomato cultivation in Kerala. Among the various diseases affecting the crop, leaf curl disease is known to cause severe loss especially during summer seasons.

A survey undertaken to know about the incidence of the ToCLV in Palakkad and Vellanikkara areas of Kerala revealed that the per cent disease incidence varied from location to location and also from season to season. Disease incidence ranged from 2 to 6 per cent and 22 to 47 per cent in Palakkad and Vellanikkara respectively in rabi season. While the survey data on summer revealed that ToLCV infection was severe ranging from 81 to 100 per cent in Palakkad and 64 to 83 per cent in Vellanikkara.

Symptoms of the disease were found to be almost similar under field and artificial conditions. The characteristic symptoms of the disease were curling and puckering of the leaves, stunted growth, reduction in leaf size and internodal length resulted in bushy appearance. The flowers were partially or completely sterile and yielded few or no fruits. In addition to the general symptoms, four different types were observed *viz.* upward curling and cupping, curling and rolling, curling with purple tint and yellowing and curling.

Transmission conducted by grafting showed successful transmission of ToLCV by producing the four types of symptoms. Among the two methods adopted, approach grafting showed high transmission rate and less incubation period compared to wedge grafting.

Studies with whiteflies showed maximum transmission (40 – 90 per cent) and minimum incubation period (16 – 18 days) in summer and all the four different types of symptoms could be successfully transmitted to the healthy plants.

Serological studies using DAC –ELISA revealed close relationship of ToLCV with *African cassava mosaic virus* and confirmed the presence of *Begomovirus* in all the four different types of samples understudy.

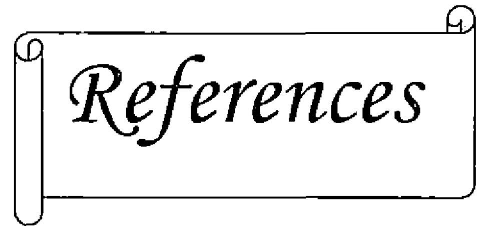
The protocol suggested by Rogers and Bendich (1994) with slight modification in spinning rate and incubation period was found to be best for extracting the DNA from ToLCV affected plants.

The presence of ToLCV in four different types of symptoms under investigation was confirmed with the PCR amplification. The primers CRv301F and CRc 1152R were found best for the PCR amplification of infected plants showing four different types of symptoms of ToLCV. The coat protein gene from the samples were amplified and sequenced. The phylogenetic analysis of the four sequences revealed that, sequences are different with respect to nucleotide sequences and upward curling and cupping type and curling and rolling type formed one clade whereas yellowing and curling type and curling with purple tint formed another clade.

The four types of symptom observed in Kerala associated with ToLCV were confirmed to be incited by begomoviruses. Yellowing and curling type and curling with purple tint type were found identical to the already reported strains of ToLCVK3 and ToLCVK5 with DNA-A components. The upward curling and cupping type and curling and rolling type showed homology to *Ageratum yellow vein mosaic virus* and *Pepper (Chilli) leaf curl Pakistan virus* respectively with respect to the coat protein region. All isolates were similar to monopartite begomoviruses with DNA-A component. The causal agent of tomato leaf curl disease in Kerala is found to be *Begomovirus* belongs to family *Geminiviridae*.

In the management studies, all the treatments were superior to control. Considering the overall performance of various treatments, the insecticide imidacloprid in combination with botanicals Perfekt or imidacloprid alone or Perfekt alone were found to be the best ones for the management of ToLCV. In addition, application of *Pseudomonas fluorescens* + Perfekt also recorded good yield and reduced the infection to certain extent. Botanicals like *Clerodendron infortunatum*, henna with 1 % milk and 2 % neem oil provided protection only at early stage of infection. This study also revealed that, the application of Perfekt alone or in combination with *Pseudomonas fluorescens* can be adopted in organic farming.

Thus the present study augmented our understanding of the various aspects of tomato leaf curl disease in Kerala specifically, the symptomatology, molecular characterisation and disease management strategies.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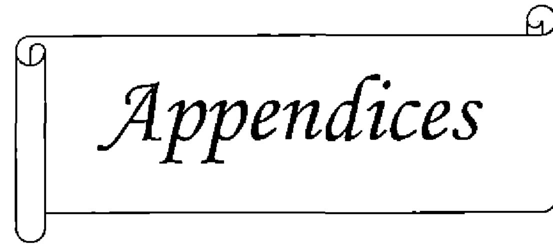
Appendices

APPENDIX – I

DAC- 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	-	1000ml
Tween 20 (0.05%)	-	0.5ml

2) Coating buffer (pH 9.6)

Sodium carbonate	-	1.59g
Sodium bicarbonate	-	2.93g
Sodium azide	-	0.2g
Water	-	1000ml

APPENDIX- II

DNA ISOLATION

1. 2x CTAB extraction buffer (100 ml)

CTAB	:	2g
Trisbase	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

3. Chilled isopropanol

Isopropanol was stored in refrigerator at 0°C and was used for the study.

APPENDIX-III

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

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

2. Loading Dye (6X)

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of Ethidium bromide (stock 10 mg/ml: working concentration 0.5 μg/ml) and was stored at room temperature in a dark bottle.

CHARACTERISATION AND MANAGEMENT OF TOMATO LEAF CURL VIRUS IN KERALA

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Tomato is one of the popular and widely grown vegetables in the world. Occurrence of diseases acts as the major constraint in the cultivation of tomato in Kerala. Recently, tomato leaf curl, a whitefly vectored gemini virus, has emerged as a serious threat to tomato cultivation.

Considering the importance of tomato leaf curl disease (ToLCD) in the state, Kerala Agricultural University has initiated research on developing resistant varieties against this disease. The present study was undertaken, giving emphasis on the molecular variability, and disease management aspects.

Survey undertaken to record the incidence of ToLCV in Palakkad and Vellanikkara revealed that, the incidence varied from location to location and also from season to season. Disease incidence was less during September to December and severe in summer season recording 74 to cent per cent in Palakkad and 64- 83 per cent in Vellanikkara.

Four different types of symptoms were seen associated with ToLCV affected plants. In transmission studies, grafting and the vector, *Bemisia tabaci* were able to transmit the disease with production of all the types of symptoms on healthy plants.

Serological studies using DAC – ELISA revealed close relationship of ToLCV with *African cassava mosaic virus* and thus confirmed the association of gemini virus in the four different types of symptoms understudy. The protocol suggested by Rogers and Bendich (1994) with slight modification was found to be best for extracting the DNA from ToLCV infected plants.

The presence of ToLCV in the four types of symptoms under investigation was confirmed with the PCR amplification with primers CRv301F and CRc 1152R. The coat protein gene from the samples were amplified and sequenced. The

phylogenetic analysis of the four sequences revealed, differences in nucleotide sequence . Yellowing and curling and curling with purple tint types symptom producing strains were found identical to the reported strains of ToLCVK3 and ToLCVK5 with DNA-A component. The upward curling and cupping type and curling and rolling type showed homology to *Ageratum yellow vein mosaic virus* and *Pepper (Chilli) leaf curl Pakistan virus* respectively with respect to the coat protein region.

In the management studies, all treatments were superior to control. Among the 16 treatments, the combined spray of imidacloprid and the botanical, Perfekt showed 80.84 per cent disease reduction and highest yield. In addition, imidacloprid alone, Perfekt alone and *Pseudomonas fluorescens* + Perfekt were also effective.

Thus, the present study augmented our understanding of the various aspects of tomato leaf curl disease in Kerala specifically, the symptomatology, molecular characterization and disease management strategies.

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