A DISSERTATION ON

Molecular Characterization and Variability Analysis of Capsicum chlorosis virus (CaCV) Infecting Tomato Fruits in India

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DEPARTMENT OF BIOENGINEERING
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IN BIOTECHNOLOGY
BY

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UNDER THE SUPERVISION OF

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DECLARATION FORM

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I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

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Certificate that **Mr. Qazi Mohammad Qubais Haq** (1700100319) has carried out the research work presented in this thesis entitled **"Molecular Characterization and Variability Analysis of Capsicum chlorosis virus (CaCV) Infecting Tomato Fruits in India"** for the award of **Dual Degree B.Tech.-M.Tech. Biotechnology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of his **Dual Degree Biotechnology.**

I wish him good luck and bright future.

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TO WHOM IT MAY CONCERN

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Date: Qazi Mohammad Qubais Haq

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List Of Abbreviations

ACLSV	Apple chlorotic leaf spot virus
AGE	Agarose Gel Electrophoresis
BIP	Backward Inner Primer
BLAST	Basic Local Alignment Search tool
BST	Bacillus stearothermophilus
BSV	Banana Streak Virus
CaCV	Capsicum Chlorosis Virus
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cucumber Mosaic Virus
CTAB	Cetyltrimethylammonium Bromide
DAC – ELISA	Direct Antigen Coated Enzyme Linked Immunosorbent Assay
DIBA	Dot Immuno-binding Assay
DNTPs	Deoxy nucleoside Triphosphate
EDTA	Ethylene-Diamine Tetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium Bromide
FIP	Forward Inner Primer
GBNV	Groundnut Bud Necrosis Virus
GMO	Genetically Modified Organisms
GRSV	Groundnut Ringspot Ortho-tospovirus
GYSV	Groundnut Yellow Spot Virus
HBV	Hepatitis B Virus
HNB	Hydroxy Naphthol Blue
IAP	Inoculation Access Period
IC - PCR	Immuno-capture Polymerase Chain Reaction
INSV	Impatiens Necrotic Spot Virus
IYSV	Iris Yellow Spot Virus

JPN-W	N Protein of WSMoV Isolates from Japan
JYMV	Japanese Yam Mosaic Virus
LAMP	Loop- Mediated Isothermal Amplification
MCT's	Micro-centrifuge Tubes
MEGA	Molecular Evolutionary Genetics Analysis
MMLV	Moloney Murine Leukemia Virus
MSA	Multiple Sequence Alignment
NASBA	Nucleic Acid-based Amplification
NCBI	National Centre for Biotechnology Information
NP	Nucleocapsid Protein
NsM	Non-Structural Movement Protein
NSs	Non- Structural Protein
ORF	Open Reading Frames
PBNV	Peanut Bud Necrosis Virus
PCR	Polymerase Chain Reaction
POC	Point of Care
PPV	Plum Pox Virus
PRSV	Papaya Ringspot Virus
PVP	Poly-Vinyl Pyrrolidone
PYSV	Peanut Yellow Spot Virus
RAPD	Random Amplified Polymorphic DNA
RdRp	RNA dependent RNA Polymerase
ROX	6-Carboxyl-X-Rhodamine
RT – PCR	Reverse Transcription Polymerase Chain Reaction
SG	SYBR Green
SSA	Sub- Saharan Africa
TAE	Tris-acetate EDTA
TAI-W	Taiwan interacted favorably with WBNV
TCSV	Tomato Chlorotic Spot Virus
TMV	Tobacco Mosaic Virus
TSWV	Tomato Spotted Wilt Virus

TYLCV	Tomato Yellow Leaf Curl Virus
UNG	Uracil-N-glycosylase
UTR	Untranslated Region
WBNV	Watermelon Bud Necrosis Virus
WSMoV	Water Melon Silver Mottle Virus
β – M.E.	β - Mercaptoethanol

1 INTRODUCTION

Some of the causes endangering food security include rapid population increase, urbanisation, and climate change. Crop production and plant yield have never been more crucial to feeding the globe. In addition to germplasm, a number of other factors affect harvest yield, preventing it from achieving its potential output. Potential yield measures how productive a crop cultivar will be under ideal water, nutritional, and biotic stress circumstances (e.g., pests and diseases) (van Ittersum *et al*., 2013). Global food output has decreased by 30–50% as a result of factors including soil nutrients, cultivation techniques, environmental factors, pests, and diseases. Researchers are developing a microbial strategy to alleviate the effects of excessive salinity, including the strains that improve fertilizer-use efficiency, in an effort to close the yield gap brought on by abiotic stress. (Singh *et al*., 2001).

Plant viruses are common and significant plant pathogens commercially. Almost all plants that people cultivate are plagued by at least one virus, whether they are grown for food, fibre, or both. The second-most significant plant pathogens after fungus are viruses. Plant viruses are thought to generate annual economic losses of over a few billion dollars worldwide (Hussain, 2016). Plant viruses interfere with the distribution of nutrients that the plant has created through photosynthesis, which causes harm inside plant cells. Because they vary by geography, virus strain, host plant cultivar/variety, and period of infection, crop damages brought on by viral infections are difficult to anticipate (Chakraborty & Newton, 2011). Viral infections are identified by symptoms such as crinkling, browning of leaf tissues, mosaic, and necrosis. However, because plant viruses may not always produce symptoms, symptoms may not always be visible. Additionally, plants may exhibit symptoms resembling viruses in response to unfavourable environmental conditions, nutritional imbalances, infection by the other pathogen groups discussed above, harm from pests or abiotic agents, among other things. Thus, viral disease detection based on symptoms more challenging than other pathogens (Savary *et al*., 2012).

Tospoviruses from the Bunyaviridae family are important viral diseases in numerous Indian crops. There is a total of 11 known tospovirus species, five of which are known to exist in India: the groundnut bud necrosis virus (GBNV), the groundnut yellow spot virus (GYSV), the iris yellow spot virus (IYSV), the watermelon bud necrosis virus (WBNV), and the capsicum chlorosis virus (CaCV). Since the 1960s, different crops from the Cucurbitaceae,

Fabaceae, Leguminosae, and Solanaceae families have been affected by tospovirus-like illnesses in India (Andrew M.Q. King, Michael J. Adams & Lefkowitz, 2012). The first distinct tospovirus species discovered as responsible for the groundnut bud necrosis disease was the GBNV in India in the beginning of the 1990s. One of the most significant plant virus families in the world, tospoviruses (family *Bunyaviridae*, genus *Tospovirus*) infect a variety of commercially significant agricultural plants (Holkar *et al*., 2017) . The Tospovirus genus' Capsicum chlorosis virus (CaCV), which was initially discovered in tomato in northern India during the rainy season in 2007. Compared to healthy plants, the infected ones grew more slowly(S. R. Kunkalikar *et al*., 2010a). Of the five tospoviruses that have been reported so far from India, CaCV, WBNV and GBNV all belong to the WSMoV serogroup (REDDY *et al*., 1992) and infect solanaceous and leguminous crops. Iris yellow spot virus (IYSV) and Peanut yellow spot virus (PYSV) are restricted to onion and peanut, respectively(Chu & Yeh, 1998).

CaCV is a known commercially significant virus that has started infecting peanut (*Arachis hypogaea*) in China as well as plants like tomato and pepper in Australia and Thailand (Knierim *et al*., 2006). The observation that both PBNV and CaCV can produce similar, albeit not identical, symptoms in tomatoes justifies further research for creating crop improvement strategies against these two tospoviruses in vegetable and legume crops in India, even though the distribution and economic impact of CaCV in that country have not yet been fully understood.

The two most important vegetable crops in India that are heavily afflicted by tospoviruses are tomato and chilli. The illnesses brought on by these viruses can result in a 100% loss of produce and frequently have negative economic and social repercussions. CaCV is a new danger to India's vegetable output in addition to GBNV and WBNV (Balol & Patil, 2014). Although numerous tospoviruses have been discovered from tomato, which is their most frequent host, it is unknown why the majority of tospoviruses infect tomato and pose a hazard to its agriculture. It is likely that many more undescribed tospoviruses may emerge as a possible hazard to vegetable farming in India given the present rate at which novel tomato-infecting tospoviruses—which were previously exclusive to East Asia and Australia—are now being detected in India. Understanding the biology and molecular characteristics of newly developing tospoviruses in India is crucial in this circumstance. According to earlier reports and observations, under unfavourable environmental conditions, CaCV mimics the signs and hosts of PBNV(Kunkalikar SR *et al*, 2010a).

Similar to GBNV, CaCV has a wide host range. They are members of the Amaranthaceae, Cucurbitaceae, Apocynaceae, Chenopodeaceae, Fabaceae, and Solanaceae families. The isolates produced similar symptoms with no differences on the indicator hosts, such as tomato and chilli, which are the two distinct naturally infected hosts. To find the thrips vector transferring CaCV to India, research is necessary(S. R. Kunkalikar *et al*., 2010b).

Tospoviruses can bring outcome in yield losses of up to 100%, depending on the crop's stage and the season(Holkar *et al*., 2019). Tospoviruses are transmitted by thrips (Thysanoptera) insects in a circulative and propagative manner. The fact that only adults who contracted the virus during the first larval stage are able to transmit the virus is a distinctive aspect of the thrips-tospovirus connection (Daimei *et al*., 2017). However, the disease incidence in this host depends on thrips infection, which picks up the virus from other crops or weed hosts in a largely monocyclic way (Manjunatha, 2008). On the leaves, fruits, and stems of the host plants, GBNV causes chlorosis, necrosis, and necrotic ring patches; infection frequently results in necrosis and plant death.. The viral particle has a tripartite (L-large, Medium, and S-small), single-stranded, ambisense RNA genome and is enclosed. Its diameter ranges from 80 to 120 nm (Holkar *et al*., 2017).

There are currently very few options for managing or curing plant viral infectious diseases in the field. Therefore, the most important step in halting the potential spread of the infectious disease is early diagnosis of the relevant causal organisms. Therefore, efficient and effective treatment of plant diseases necessitates the adoption of specific and sensitive technologies for accurate identification. Viral diagnostics is a field where novel approaches and procedures are constantly being developed and used. There are numerous ways that have been developed to identify plant viruses, including genetic techniques, serological techniques, and microscopical examination (López *et al*., 2009).

In Molecular techniques such as polymerase chain reaction (PCR) can be utilised to overcome that type of difficulties and boost sensitivity and specificity of pathogen detection. For the detection and naming of the diseases, specific PCR assays have been created (Bertolini *et al*., 2007). Despite the availability of multiple diagnostic techniques, there isn't a single quick, accurate, low-cost, and labour-intensive technique for diagnosing such diseases in the field. Although PCR approaches have substantially improved our capacity to identify Tospovirus infection in plants, their need for a highly accurate thermal cycler has prohibited their widespread use as a standard diagnostic tool in the field or by

private clinics. Alternative isothermal nucleic acid amplification techniques that simply need a basic heating equipment have been created to provide workable platforms for quick and accurate target nucleic acid detection. These include ramification amplification, loop-mediated isothermal amplification, and nucleic acid-based amplification (NASBA) (Notomi, 2000). The exceptional gene amplification method known as loop-mediated isothermal amplification (LAMP), which allows one kind of enzyme to perform the reaction at a constant temperature, is distinguished from other genetic tests by its speed and simplicity. Without the use of any special reagents, the LAMP method may amplify a small number of DNA copies to a large number in less than an hour (Tomita *et al*., 2008). The strand displacement reaction is used in this method, which is characterised by utilising 4–6 different primers that are each intended to detect 6–8 separate areas on the target gene. The reaction process occurs at a constant temperature (60–65 °C) and is finished in 60 minutes (Notomi, 2000). Additionally, the entire LAMP test is carried out in a single reaction tube under isothermal conditions, from amplification to detection.

The goal of this research was to detect and characterize Capsicum chlorosis virus at molecular level. Hence, the present studies have been undertaken with the following objectives.

Objective of the Study:

1. Molecular characterization of Capsicum chlorosis virus infecting tomato fruit
2. Variability analysis of Capsicum chlorosis virus based on Np gene

2 Review Of Literature

To grasp the significance of this research, it's required to first grasp an overview of plant viruses and disease detection technologies for disease management to avoid substantial losses in agricultural production by reducing both the number and quality of food crops. Viruses have long posed a significant threat to a wide range of crops, with viral infection accounting for approximately 40% of overall crop losses. Virus is the second most significant pathogen, after only fungi. Plant viruses from more than 25 different families have infected a wide range of crop plants. Tospoviruses of the family *Bunyaviridae* are very significant viral pathogens in several crops in India. The current chapter provides a critical examination of frequently employed methods for quick, accurate, and sensitive detection of tospoviruses and summarises the tospovirus research work done in India over the past more than fifty years.

2.1 Plant viruses:

Numerous socioeconomic issues, including as climate change, rapid population expansion, food insecurity, and environmental degradation, are currently plaguing the world. These issues have an adverse impact on farming practises, the health of people and animals, and vulnerable ecosystems everywhere. At the same time, the world is entering a fascinating period of unparalleled scientific and technical advancements that show enormous promise for resolving these issues (Tscharntke *et al*., 2012) .

A significant contributor to plant disease, viruses are thought to have a yearly economic impact of over $30 billion. They are roughly 50% of the pathogens that cause new and re-emerging plant diseases globally, and they harm both cultivated plants and wild vegetation. Virus diseases pose a hazard to cultivated plants by reducing their development and vigour, which lowers gross yields, as well as by compromising produce quality, which lowers marketable yields. Globally, these crop losses result in damage ranging from minor to complete failure (Tscharntke *et al*., 2012). The world's tropical regions, where agriculture is the primary source of income for the majority of people, are frequently where their economic and sociological effects are most severe. This is especially evident in developing countries' subsistence agriculture (Anderson *et al*., 2004). By changing the species makeup of plant communities and triggering genetic erosion, virus infections harm natural ecosystems by potentially causing the extinction of species. Controlling viral disease epidemics is becoming more challenging due to increased climate instability brought on by

global warming (Cooper & Jones, 2006). The prevalence of viral illnesses is increasing the hazards to farming systems because plant viruses are so common and there are no direct treatments for them. To provide a comprehensive knowledge of the causes causing their epidemics, a systems biology approach is therefore necessary.

Three major plant virus families, the Begomoviruses, tospoviruses, and potyviruses—transmitted by whiteflies, thrips, and aphids, respectively—threaten food security by wreaking havoc on tropical and subtropical food crops(R. A. C. Jones, 2009) .

The tospoviruses *Tomato spotted wilt virus* in peanut (groundnut), pepper, and tomato globally and *Peanut bud necrosis virus* in tomato and peanut in south and Southeast Asia are examples (Rey & Vanderschuren, 2017). Additionally, potyviruses frequently trigger synergistic interactions that intensify disease symptoms when present in mixed virus infections, leading to fatal diseases that jeopardise food security, such as sweet potato virus disease brought on by a combination of Sweet potato chlorotic stunt virus and potyviruses like Sweet potato feathery mottle virus and maize lethal necrosis disease brought on by a combination of Maize chlorotic mottle virus and potyviruses (Mahuku *et al*., 2015).

2.2 Tospovirus, Family Bunyaviridae

Tospoviruses (family Bunyaviridae, genus Tospovirus) are enveloped isometric RNA viruses with a tripartite genome containing small (S), medium (M), and large (L) segments of ssRNA. Among all the viral diseases, Tospoviruses are rising as a key limiting factor to produce crops in India (Holkar *et al*., 2017). The genus Tospovirus of the family Bunyaviridae is composed of 19 species described so far, and of them 14 have been identified from Asia. Tospoviruses are not seedborne, and it is thought that the primary mode of transmission is by thrips from other crops or weeds, with secondary transmission occurring from infected plants within the same field (Pappu *et al*., 2009). A unique aspect of the thrips-tospovirus relation is that only the adults that acquire the virus in their early development / first larval stage can infect the crops with viral infection. GBNV is one of the most commercially important plant viruses in the world, belonging to the Tospovirus genus and Bunyaviridae family. GBNV can reduce yields by up to 100% depending on the crop growth period and season affecting a variety of crops such as peanut, potato, tomato, soybean, urdbean, mungbean and cowpea (Holkar *et al*., 2017).

2.3 Historical Perspective:

Since the 1960s, disease symptoms similar to those caused by tospoviruses have been reported on several crops in India, including black gramme (*Vigna mungo*), brinjal (*Solanum melongena*), chilli (*Capsicum annuum*), cowpea (*Vigna unguiculata*), groundnut/peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), pea (*Pisum sativum*), potato (*Solanum lycopersicum*) (Basavaraj *et al*., 2017) .

Up until 1990, the tospovirus genus was monotypic, with the only species being the tomato spotted wilt virus (TSWV), and tospoviral infections were initially believed to be brought on by TSWV. The groundnut bud necrosis virus was given that name in 1992 when it was determined by serology that a tospovirus other than TSWV was responsible for the groundnut bud necrosis illness. Based on the nucleocapsid protein (N) gene sequence, GBNV was further validated as a unique tospovirus (REDDY *et al*., 1992).

Later, the Peanut Yellow Spot Virus (PYSV), a different tospovirus that causes yellow spots on groundnuts, was discovered (Satyanarayana *et al*., 1998). Watermelon bud necrosis virus (WBNV), a unique tospovirus, was discovered to be linked to this novel and rare illness in 1991 and 1992 in portions of southern India. The disease is characterised by leaf mottling and shoot dieback. The most recently reported tospoviruses in India include *Iris yellow spot virus* (IYSV) on onion (*Allium cepa*)(Ravi *et al*., 2006) and garlic (*Allium sativum*) (Gawande *et al*., 2010) , and *Capsicum chlorosis virus* (CaCV) on tomato and chili (*Capsicum annum*) (S. R. Kunkalikar *et al*., 2010b).

2.4 Geographic Distribution and Economic Impact:

The tospoviruses in India are spread in a wide range of geographical areas. While its presence in other states cannot be ruled out, PYSV has been recorded from Andhra Pradesh. There are no known tospovirus outbreaks in the north-eastern states of India (A.M., 2007).

GBNV, followed by WBNV and other tospoviruses, is the most significant tospovirus in India economically. In India, GBNV resulted in a 70–90% loss in groundnut production(Becker *et al*., 2015). Different tomato-growing regions in Maharashtra, Karnataka, and Andhra Pradesh reported serious outbreaks of GBNV, with up to 100% disease prevalence being observed from 2003 to 2006 (Rao & Reddy, 2020). Potato losses of up to 29% have been reported as a result of disease stem necrosis brought on by GBNV, in addition to losses in groundnut and tomato. An outbreak of WBNV in certain regions of

southern India greatly hampered watermelon cultivation, prompting farmers to give up the crop altogether(Jain et al., 2005). The impact of IYSV and CaCV on onion and tomato, respectively, in the country is not known, although yield reduction was anticipated due to severe and widespread incidence of the disease in several parts of Maharashtra (S. R. Kunkalikar *et al*., 2010b).

2.5 Diseases and Virus Characterization:

More than 20 tospoviruses have been reported from all over the world (Mandal *et al*., 2012) . To date, five tospoviruses, WBNV, CaCV, IYSV, PYSV, and GBNV, are known in India.

2.5.1 Watermelon bud necrosis virus:

In southern India, watermelon was first shown to be infected with WBNV in 1991. This dangerous pathogen affects watermelons and other cucurbitaceous plants, where incidences can range from 39 to 100 percent and yield losses can range from 60 to 100 percent. Based on the virus's host range, symptoms, transmission, and serology, it was initially determined to be a new tospovirus attacking watermelon (S. Kunkalikar, Poojari, & Rajagopalan, 2007).

Plant hosts that act as ancillary hosts for the virus' persistence include *Parthenium hysterophorus*, Amaranthus species, *Euphorbia heterophylla*, *Malvastrum coromandelium*, and *Abutilon indicum* (S. R. Kunkalikar *et al*., 2011).

Thrips flavus was said to be the vector for WBNV in India. Based on species-specific markers for mitochondrial mtDNA and subsequent deep sequencing, Thrips palmi has recently been identified as a WBNV vector (Hogenhout *et al*., 2008) .

The field symptoms of WBNV in watermelon initially develop as chlorotic mottling, yellow spots or patches, and mild crinkling of leaves. Subsequently, necrosis of buds in the growing tips results in dieback of vines . In the young crop, rapid dieback and wilting of plants develop dramatically causing a total loss in the affected plants. In the mature crop, shortened internodes, upright growth of younger shoots, necrosis on stem, petiole, and fruit stalk are commonly seen. Infected plants produce unmarketable small, deformed fruits with uneven surface, and necrotic or chlorotic rings, depending on the cultivar (S. Kunkalikar, Poojari, & Rajagopalan, 2007).

2.5.1.1 Serological Relations:

Based on the experiment with PAbs, WBNV is serologically indistinguishable from GBNV and CaCV, indicating that it belongs to serogroup IV (McMichael et al., 2002). PAbs to the N protein of WSMoV isolates from Japan (JPN-W) and Taiwan interacted favourably with WBNV (TAI-W). The mild response of the PAbs to the N protein of the Indian groundnut isolate's GBNV, however, suggested a close serological connection. however, failure to respond to based on the PAbs to the N protein of TSWV and INSV, the WBNV is different from or independent of these tospoviruses (Jain *et al*., 2005).

2.5.1.2 Molecular Biology:

WBNV genome has not been adequately characterized. Li and his colleagues created the whole genomic sequence of the WBNV virus (2011). The WBNV-JT isolate's L RNA is 8916 nucleotides long, with 5′- and 3′-UTRs measuring 247 and 32 nucleotides, respectively (Li *et al*., 2011). The L RNA has a negative polarity and a coding sequence of 8637 nucleotides that produces the 332 kDa RdRp protein, which has 2878 amino acids. The Gn/Gc glycoprotein precursors of 127.15 kDa (1121 amino acids) and NSm protein (34.10 kDa; 307 amino acids) are encoded by the M RNA genome, which is 4794 nucleotides long and has two ORFs in an ambisense orientation. An IR of 412 nucleotides separated the two ORFs (Kumar *et al*., 2010; Li *et al*., 2011). The WBNV N gene (accession number AF045067) and the GBNV and WSMoV N genes had 81 and 79 percent nucleotide identical and 82 and 84 percent amino acid identity, respectively. The nucleotides identities among the 28 field isolates of WBNV whose N gene nucleotides sequences were identified and analysed in India in 2011 varied from 94.1 to 100 percent utilising pairwise sequence alignment (S. Kunkalikar, Poojari, & Rajagopalan, 2007).

2.5.2 Capsicum chlorosis virus:

CaCV was initially discovered and described from Australia on tomato and chilli (McMichael *et al*., 2002). CaCV is recently reported to be prevalent in northern, central, and southern parts of India.

In Thailand, the thrips Ceratothripoides clartris were identified as the CaCV vector (Premachandra *et al*., 2005) . Whereas in Australia, CaCV was shown to be transmitted by *Frankliniella schultzei* and *Thrips palmi*(Riley *et al*., 2011) . It is necessary to conduct research to determine the thrips vector in India that transmits CaCV.

CaCV in tomatoes and chillies causes symptoms that are almost identical to GBNV symptoms. On immature chilli leaves, CaCV first causes yellow spots or patches that eventually unite to produce a mosaic pattern (Mandal *et al*., 2012). Later, small concentric rings develop around and inside the adult leaves' yellow spots, causing the leaves to distort. The plant with the virus grows tiny leaves with a thin canopy and exhibits apical necrosis. Frequently, necrotic streaks on delicate stem parts can also see, as in GBNV infection cases. On tomatoes, necrotic and chlorotic Ringspots appear on fruits and foliage. Later, the systemic infection causes young developing tips to necrotize and then buds to do the same. fruit of a tomato with CaCV infection. Plants have surface discolorations with circular, concentric ringspots (S. Kunkalikar, Poojari, & Rajagopalan, 2007).

2.5.2.1 Serological Relations:

CaCV, WBNV, and GBNV are all members of serogroup IV of the WSMoV virus (Yeh, 1995). Numerous CaCV isolates from various hosts and locales in India were examined serologically to determine their serogroup IV membership (S. R. Kunkalikar *et al*., 2010b; Mandal *et al*., 2012).

2.5.2.2 Molecular Biology:

In 2008, the N gene sequence was used to record the first CaCV genome sequence in India (S. R. Kunkalikar *et al*., 2010b). Only 22 sequence accessions existed in the NCBI database as of 2015. Only one Indian isolate of CaCV has the full sequences for all three genome segments (S, M, and L) to yet (Basavaraj *et al*., 2017; S. R. Kunkalikar *et al*., 2010b). The N and NSs genes' two non-overlapping ambisense ORFs were separated by an IR of 824 nucleotides in the 3105 nucleotides that made up CaCV's S-RNA genome. The Indian strain's IR was discovered to be 372 nucleotides shorter than the isolate from CaCV-Thailand. Similar to the NSm and Gn/Gc genes, the M-RNA genome is 4821 nucleotides long and has an IR of 425 nucleotides. The RdRp gene, which had 8634 nucleotides, was located within the L segment, which had 8912 nucleotides(S. R. Kunkalikar *et al*., 2010b) .

2.5.3 Iris yellow spot virus:

IYSV is an important constraint in onion and garlic in several countries(Pappu *et al*., 2009) . The virus was originally discovered in the USA in 1989, and after that, it was reported in

other nations. IYSV was initially discovered in India in 2006 on onions (*Allium cepa*), garlic (*Allium sativum*), and chives (*Allium tuberosum*) (Hall, 1993) .

The IYSV's native hosts in India are the onions, garlic, and garlic chives. The D. stramonium, *C. amaranticolor, D. metel, D. alba,* and *N. rustica* species are among the experimental host species. Thrips circulate and spread IYSV in a contagious manner. Despite the fact that T. tabaci seems to be the main IYSV vector in the USA (D. R. Jones, 2005) . The virus can only be acquired by larval stages L1 (first instar) and L2 (second instar), although all three stages (L1, L2, and adult) can transmit the virus. Changes in transmission effectiveness were the variations in T. tabaci populations, which have been connected to the mitochondrial haplotypes are present (Gawande *et al*., 2010).

On the leaves and flower-bearing stems, IYSV symptoms include chlorotic spindle- or diamond-shaped lesions (scapes). Large necrotic patches emerge on leaves and scapes as the disease spreads and the lesion's size grows. IYSV affects both seed and bulb onion crops, but the losses are more noticeable in the seed crop because necrotic spots on the flowers and seeds cause the stalks to topple over too soon, resulting in reduced seed production. Straw coloured, spindle-shaped patches on garlic and garlic chives with less defined ends are among the symptoms. Spots combine to form larger patches as the infection spreads. These signs were: older leaves are more observable(Gawande *et al*., 2010; Ravi *et al*., 2006) .

2.5.3.1 Molecular Biology:

The virus has been characterized at molecular level in India based on N gene sequence a decade ago(Ravi *et al*., 2006) . There are 44 N gene sequences included in the Indian IYSV isolates' sequence entries in the GenBank database. However, there is no data available for the other IYSV genetic segments. The N gene sequence was primarily derived from onion (44 accessions), with only one accession each from garlic and chives, despite the fact that IYSV is known to infect these plants. Recently, the M- and S-RNA genome nucleotide sequence of an Indian IYSV isolate was determined (Gawande *et al*., 2010). The largest number of recombination events were observed in the IR of the S- and M-RNA of the IYSV genome during research on the potential involvement of recombination in the evolution of tospoviruses(Gawande *et al*., 2010; S. R. Kunkalikar *et al*., 2010b) .

2.5.4 Peanut yellow spot virus:

PYSV was discovered as a unique tospovirus in groundnut based on thrips transmission, host range, and serology (REDDY *et al*., 1992). PYSV disease in groundnut is characterised by yellow spots followed by necrosis on the leaves. PYSV has been seen in southern India with rates of up to 90%, however the impact on yield loss is unknown. PYSV can be distinguished from TSWV, INSV, and GBNV serologically. Based on S RNA sequence features, PYSV was proposed as a novel species (Reddy & Thirumala-Devi, 2003) . PYSV's N and NSs proteins differ greatly from those of other tospoviruses, with low sequence identity of 24 to 28 percent and 16 to 21 percent with the corresponding genes of known tospoviruses, respectively(Mandal *et al*., 2012) .

2.5.5 Groundnut bud necrosis virus:

GBNV was first identified on groundnut as TWSV before being renamed GBNV because to its unusual serological characteristics (REDDY *et al*., 1992; Reddy & Thirumala-Devi, 2003). Following that, the virus was discovered on multiple plant species from various geographical places, indicating that it is widespread in India (Jain *et al*., 2005).

The groundnut GBNV field symptoms are extensively reported. Mild chlorotic patches emerge first on new quadrifoliate leaves, followed by necrosis and chlorotic rings. The key distinguishing feature of the monsoon season is necrosis of the terminal bud. Secondary symptoms include stunting, axillary shoot growth, and leaflet deformity. Plants that become infected early become bushy, stunted, and die prematurely. When plants older than one month become infected, the symptoms are limited to a few branches (Reddy & Thirumala-Devi, 2003) .

2.5.5.1 Clinical signs and histological observations/ Symptoms or Signs

2.5.5.1.1 In peanut:

Mild chlorotic patches emerge first on new quadrifoliate leaves, followed by necrosis and chlorotic rings. The predominant sign in wet and post-rainy seasons is necrosis of the terminal bud. Secondary symptoms include stunting, axillary shoot growth, and leaflet deformity. (Mandal *et al*., 2012).

2.5.5.1.2 In tomato

Necrotic rings on the leaf, stem necrosis, concentric rings, and uneven colour on the fruit are all symptoms(Mandal *et al*., 2012) . Necrosis of the leaves frequently results in the collapse of a stem or the entire plant, simulating blight symptoms. In general, tomato plants that become infected at an early stage frequently collapse and eventually dies (Akram & Naimuddin, 2010a).

2.5.5.1.3 In potato

Infection symptoms include stem/petiole necrosis, foliar spotting/deformation/necrosis, and plant stunting (Kaushal *et al*., 2010). Foliage necrosis frequently results in the collapse of a stem or the entire plant. In general, potato plants that become infected at an early stage frequently collapse and eventually dies (Akram & Naimuddin, 2010b).

2.5.5.2 Serological Relations:

Polyclonal antibodies (PAbs) were developed against TSWV pure virions in the 1960s and 1970s for serological detection of the virus(Nigam *et al*., 2019) . On the basis of nucleocapsid (N) protein serology, GBNV, WBNV and CaCV were grouped in WSMoV serogroup (Yeh and Chang). Because tospovirus purification is challenging, recombinant N protein generated in Escherichia coli has been used to produce PAb on a sustainable basis, leading to the commercialization of an ELISA-based diagnostic kit for GBNV(Jain *et al*., 2005).

2.5.5.3 Molecular Biology Of GBNV

2.5.5.3.1 Genome Sequence Resource

Based on the NCBI database for GBNV in India indicated that, of the three genome segments of GBNV, the S RNA (RNA 3 segment) is extensively investigated, with 206 out of a total of 263 accessions (78.3 percent) by 2015. The NCBI GenBank database has 400 tospovirus accessions, with GBNV accounting for 263 of them (65.75 percent). This suggests that GBNV is the most researched tospovirus in India. In 2002, the first GBNV sequencing data were deposited in GenBank (Lokesh *et al*., 2010). In 2011, the database had up to 75 sequencing entries for the N gene. The N gene is the most investigated of the five genes in the three GBNV genome segments, with 204 entries in the NCBI GenBank, followed by movement protein (NSm) with 48 entries.

2.5.5.3.2 Structural Genomics

The entire genome of GBNV (type isolate, groundnut) was sequenced, and it consisted of three single-stranded RNA molecules wrapped with nucleocapsid protein: large (L, 8.9 kb), medium (M, 4.8 kb), and short (S, 3.05 kb) (N) (Satyanarayana *et al*., 1998).

The L RNA is negatively polarised, encoding the 330 kDa L-protein viral polymerase (2877 aa) (Gowda *et al*., 1998). In the viral sense, the M RNA encodes a 34.3 kDa NSm protein and a 127.3 kDa precursor to the two viral membrane glycoproteins, Gn and Gc, in the viral complementary sense. In the virus sense, the S RNA encodes a 49.5 kDa NSs protein and a 30.6 kDa N protein in the virus complementary sense (Satyanarayana *et al*., 1998). Comparative sequence study of GBNV isolates from groundnut, mungbean, and tomato indicated that the M RNA genome differed significantly in intergenic regions (56-89 percent sequence identity) and Gn/Gc protein.

So far, genetic diversity research on GBNV have primarily focused on the N gene. The amino acid sequence identity (>90%) of the N gene is regarded as an important requirement for tospovirus species delineation (Gowda *et al*., 1998). The N gene sequences of more than 200 GBNV isolates from various hosts and places are available in GenBank. GBNV isolates from Kerala, Uttar Pradesh, and Delhi were almost identical (99 percent identity) to the GBNV type isolate (Jain *et al*., 2005).

2.5.5.4 TRANSMISSION:

Mechanical transmission:

In general, independent the diseased host's or viral isolate, signs of CaCV infection, such as bud necrosis, lesions, mottling, stunted growth, chlorosis, and necrotic rings, are quite similar. The virus was successfully transmitted using sap injection. Mechanical inoculation was accomplished by grinding young infected leaves in a cold 0.05M phosphate buffer (pH 7.0) with 0.02 M, -mercaptoethanol(Walter & Barr, 2011).

In mechanical transmission of GBNV to cowpea and groundnut, Reddy *et al*. (1992) utilised 0.05 M potassium phosphate, pH 7.0, containing either 0.02M -mercaptoethanol or 0.75 percent (v/v) mono-thioglycerol(Manjunatha, 2008) .

A 0.05M phosphate buffer with a pH of 7.0 and 0.75 percent thioglycerol was employed to transmit GBNV isolates from leaf curl afflicted mung bean and urd bean to cowpea and the appropriate host species(Sastry *et al*., 2019).

0.1M phosphate buffer, pH 7.2, containing 0.1 percent (v/v) -mercaptoethanol, was used to mechanically transfer the soybean isolate to cowpea. Plant predisposition can be improved by keeping them in the dark for 24 hours prior to inoculation (Manjunatha, 2008).

Thrips transmission:

Thrips are the only in a circulative and propagative way. Tospovirus is conveyed in the thrips body via midgut and salivary epithelium membrane barriers to the salivary gland or via ligaments linking the midgut and the salivary gland(Ghosh *et al*., 2017) . Of 11 species of thrips recorded as vectors of tospoviruses throughout the world, five thrips vectors, *Ceratothripoides claratris*, *Frankliniella schultzei*, Scirtothrips dorsalis, *Thrips palmi*, and *T. tabaci*, have been described as tospovirus vectors worldwide. Thrips palmi, *T. alis*, and *T. tabaci* have all been reported from India. The distribution of thrips genera in vegetable crops investigated in Andhra Pradesh, Karnataka, and Maharashtra from 2006 to 2007 revealed a preponderance of *Thrips* sp. in onion, *Scirtothrips* sp. in pepper, and *Frankliniella* sp. in tomato (Pappu *et al*., 2009) .

T. palmi is assumed to be a vector for GBNV and CaCV in India (Mandal *et al*., 2012). *Citrullus lanatus*, *Cucumis sativus*, *Capsicum annuum*, *Cucurbita spp*., *Glycine max*, *Helianthus annuus, Cucumis melo, Nicotiana tabacum, Pisum sativum, Gossypium spp., Sesamum indicum, Solanum tuberosum, Solanum melongena*, and *Vigna unguiculata* have all been. After a minimum inoculation access period (IAP) of 30 minutes, a single adult thrips propagated the virus. As IAP climbed, so did the percentage of transmission (Gopal *et al*., 2010). While there is no evidence of western flower thrips (*Frankliniella occidentalis*) in the subcontinent, measures to contain this vector and the tospoviruses that could be transmitted must be devised in the event that this key thrips vector is imported. Transmission is the consequence of several processes that begin with virus ingestion on infected plants and end with virus transmission to a healthy plant. These occurrences correlate with the thrips' host-finding and feeding activity. The effectiveness with which the virus can be acquired and then transferred, as well as the shorter the latent period, can be used to quantify the ratio at which thrips become viruliferous and transmit. The

dynamics of this organism are the result of complicated interactions between plants, vectors, and viruses (Kumar *et al*., 2010; Manjunatha, 2008).

2.6 Detection Techniques for Tospoviruses:

2.6.1 Symptomatology:

It is frequently possible to identify an illness with a viral origin by looking for a typical viral symptom in plants. Tospovirus diagnostics received considerable attention during the last two decades in India. This has led to the identification of various tospovirus species. (Iwaki, 1984; R. a. C. Jones, 2005; S. Kunkalikar, Poojari, & Rajagopalan, 2007; Mahuku et al., 2015; REDDY et al., 1992).

2.6.2 Microscopy:

One of the most well-known methods for detecting viruses in plant tissues is microscopic detection using modern light and high-resolution electron microscopes. The knowledge of the viral particle's size, shape, and any surface properties is the most basic condition for virus detection. Tospoviruses are the only plant viruses that are spherical and enclosed; hence, members of this group can be easily recognised using electron microscopy of leaf. (Black *et al*., 1963; Ie, 1971).

2.6.3 Serological methods:

2.6.3.1 Enzyme Linked Immunosorbent Assay (ELISA):

Due to their immunogenic properties, plant viruses have been exploited in both serological virus detection and as antigens for the generation of antibodies. Instead of relying on biological characteristics like host range and indicator host, which could be mistaken with symptoms generated by other viruses, such as serological techniques like immuno diffusion and enzyme linked immuno sorbent assay (ELISA) are employed to identify viruses (Adams & Clark, 1977; Webster *et al*., 2004). Gonsalves and Trujillo's creation of high-grade polyclonal antisera and invention of an enzyme linked immunosorbent assay (ELISA) marked a watershed moment in Tospovirus detection and diagnosis. Currently, this serological test is the go-to procedure for diagnosing and finding Tospoviruses in plants and thrips (Gonsalves, 1986).

Using polyclonal and monoclonal antibodies, tospovirus detection by ELISA suggests at least three serogroups and four serotypes (de Avila *et al*., 1990; Gonsalves, 1986). The

association of GBNV in black gramme, cowpea, green gramme, and soybean was validated based on DAC-ELISA. Other illnesses that were once thought to be caused by TSWV, such as tomato spotted wilt and mung bean and urd bean leaf curl, were precisely identified as GBNV using the ELISA approach(Balol & Patil, 2014).

Because tospoviruses share epitopes on their single capsid protein, they are serologically related. Serological relationships between viruses may suggest that one or more of the three main structural proteins (N, G1, and G2) were serologically connected, with little to no relationship or a sharing of epitopes amongst the other structural proteins. In most cases, the relationships were established using polyclonal antibodies developed to fight infectious virions. (German *et al*., 1992).

An isolated Tospovirus Recently, Tospo-To, a tomato-borne virus, was discovered in Taiwan and was found to be serologically linked to WSMoV (Taiwan), WSMoV (Japan), and PBNV (India)(Manjunatha, 2008) . The N protein in all three isolates is similar and greater than that in other tospoviruses. Comparisons between the N protein amino acid sequences of several Tospoviruses species revealed that WSMoV and Tospo-To share 32% and 98% similarity, respectively. With TSWV and TCSV at a rate of 26%, GRSV at a rate of 33%, and INSV at a rate of 30% (Heinze *et al*.,1995).

In comparison to TSWV and INSV, WSMoV and PBNV have a N protein of 32 KD, indicating that these two viruses are closely related. They also share a high level of serological cross reactivity(Manjunatha, 2008) . The WSMoV and TSWV N proteins share 86% and 30% of their amino acid sequences, respectively, in the PBNV N protein. Furthermore, WSMoV causes severe systemic symptoms in the majority of cucurbit species but does not infect peanuts, whereas PBNV infects cucurbits locally but infects peanuts systemically (Karjalainen *et al*., 1987). Despite the fact that there is a stronger serological link between the two N proteins, it appears that serogroup IV viruses WSMoV and PBNV are two strains of the same species(Adam *et al*., 1993).

The poly antiserum was found in all the various plant components and the absorbance values (A 405 nm) ranged from 0.01 in the leaf and petiole samples from typical symptomatic tomato plants(Jain *et al*., 2005).

2.6.4 Molecular detection based on viral nucleic acid:

Techniques based on the identification of viral-specific nucleic acids are helpful for diagnostic purposes and can lessen issues with the detection of serologically unique isolates in addition to serology and symptom manifestation. For diagnostic applications, riboprobes (labelled synthetic RNA transcripts) and cDNA probes are available(Mumford *et al*., 1994). Although these methods are precise and sensitive (around 1 pg of viral RNA may be identified), their application is constrained by high costs and the need for specialist facilities to employ radioisotopes. The adoption of these approaches could be accelerated by the creation of nonradioactive nucleic acid detection methods.

2.6.4.1 PCR based detection:

PCR based techniques are extremely sensitive and provide additional advantage that the amplican can be sequenced to know more about the molecular details(Manjunatha, 2008). Various PCR-based approaches for TSWV screening have been designed; Mumford *et al*. produced the first PCR-based test (1994)(Mumford *et al*., 1994) . Nolasco *et al*. (1993) and Weekes *et al*. (1996) developed immunocapture (IC) PCR and reverse transcription (RT-PCR) for the detection of tospoviruses, respectively. Multiple universal primer combinations have been developed to perform PCR testing on infected plants and thrips to identify and characterise different tospoviruses (Nolasco *et al*., 1993).

Seven different tospoviruses were amplified using degenerate primers created from conserved sections of L genes from TSWV, water melons silver mottle virus (WSMoV), impatiens necrotic spot virus (INSV), and GBNV isolate(Chu *et al*., 2001).For the molecular identification of GBNV and WBNV isolates in India, degenerate primers were created based on the conserved nucleotide homology of the NP gene sequence from GBNV and WSMoV(Jain *et al*., 2005).

TSWV detection uses a complementary DNA (cDNA) probe designed for viral RNA(Iwaki, 1984) . In order to quickly and accurately duplicate the TSWV sequence from peanuts, reverse transcription polymerase chain reaction (RT-PCR) was used. Isolates from peanut root and leaf tissue that had less than 80% similarity to all other known viruses were categorised as unique viruses (Jain *et al*., 2005). The sero group includes viruses that have serological similarity. According to reports, TSWV and the serologically different INSV, TCSV, and GRSV have very little diversity and exhibit a high amount of cross reactivity.

The higher degree of amino acid homology between the N proteins of TCSV and TSWV and GRSV and TSWV, with 76% and 78% homology, respectively, can be attributed to the stronger degree of relatedness. The amino acid homology between the N proteins of TCSV and GRSV is 81% (Jain *et al*., 2005) .

Peanut bud necrosis virus (PBNV), initially reported to be caused by TSWV(Renuka *et al*., 2020)however, the causal agent has since been recognized as a serologically separate Tospovirus and given a specific nomenclature PBNV (Jain *et al*., 2005; Reddy & Thirumala-Devi, 2003). In contrast to TSWV and INSV, which do not systemically infect members of the cucurbitaceae, watermelon silver mottle virus (WSMoV) is now thought to be the cause of the watermelon silver mottle virus that was first described as TSWV-W from Japan and Taiwan (Iwaki, 1984; Yeh, 1995).

When the nucleic acid sequences of all the Tospovirus members were examined, the nucleotide sequence of the WSMoV N gene was identified. This revealed a nucleic acid sequence homology of only about 55%, and even less (42–46% at the amino acid level) (Yeh, 1995). According to a report, RT-PCR analysis was used to detect the presence of Tospovirus in the thrips vector *Scirtothrips dorsalis*. Similar to this, RT-PCR testing on tomato plants infected with GBNV produced DNA amplification similar to the Coimbatore isolate's N gene (830 bp). A dendrogram created using the RAPD similarity matrix showed that the *Frankliniella schultzei* population from cowpea and sun hemp only shared 50% of the similarities with the *S. dorsalis* population from tomato, groundnut, and chilies. With lowest similarity indices (0.464), Thrips tabaci from cotton GBNV was distantly connected to *S. dorsalis* and *F. schultzei* (Meena *et al*., 2005).

It was reported that the N-gene was amplified using certain primers to confirm the involvement of a Tospovirus in the necrosis of tomatoes. The N-gene from tomato samples taken from symptomatic host plants was effectively amplified by RT-PCR, suggesting a link between a GBNV isolate and tomato necrosis disease (Yeh, 1995).

2.7 Limitations:

The ELISA is the most used serological method because it is straightforward, sensitive, and suitable for use in even small laboratories. The ELISA or comparable serological methods, however, are not always sufficient for testing. For instance, some viruses that are poorly immunogenic or difficult to purify, present in low concentrations in plant tissue, such as

the barley yellow dwarf virus and the potato leafroll virus, are difficult to detect using serological approaches. Serological diagnosis is also hard for some viruses that have a broad host range (like the cucumber mosaic virus), are unstable, or have satellite RNA that is enclosed by the related virus' coat protein (Basavaraj *et al*., 2017). The smallest known plant pathogen, viroids lack a protein covering, making them immune to detection by immunological techniques.

Nucleic acid hybridization has been widely tested as a means of diagnosing various plant virus diseases (Hull 1986). However, there are still many problems limiting its wide diagnostic application both in medical and agricultural fields. One serious limitation is the use of radioactively labelled recombinant DNA probes.

2.8 The requirement for a practical tool for plant disease diagnostics and the development of LAMP:

Because of climate change, plant diseases and their management are getting increasingly challenging. New tool(s) capable of early detection and monitoring of diseases are required. The following requirements must be met by the new tools: (1) high sensitivity, (2) high specificity, (3) low per-test cost, (4) simple, (5) quick, and (6) instrument-light (Almasi, 2015). Although PCR-based detection approaches offer high sensitivity and specificity, they fall short on other criteria like as speed and device affordability. The loop-mediated isothermal amplification (LAMP) technology, which is based on isothermal amplification of nucleic acids, meets these conditions. (Notomi *et al*., 2000) .

2.9 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP):

The progress of molecular diagnosis methodologies has resulted in various enhanced technologies, such as loop-mediated isothermal amplification (LAMP). It is currently a well-established approach used in a variety of industries, including medicine, agriculture, and the food business, because to its simplicity, specificity, speed, and low-cost efforts.

Notomi and colleagues developed LAMP, a nucleic acid amplification method that allows them to amplify a specific DNA section of the hepatitis B virus (HBV) in isothermal circumstances (Notomi, 2000). This method enabled the quick, sensitive, and specific identification of a single target, opening up new diagnostic possibilities. Since the publication of this paper, LAMP has grown in popularity as a viable alternative to PCR-based approaches. (Mayboroda *et al*., 2018).

The invention of the LAMP methodology was motivated by a desire to address some of the shortcomings of traditional PCR, a process that necessitates the purchase of a high-cost piece of equipment known as a heat cycler. Because of the need for high precision in heating/cooling ramps and temperatures, there are times when the sufficient specificity for identifying the targeted targets is lost(Mayboroda *et al*., 2018). Furthermore, the polymerase enzyme is extremely susceptible to inhibitors found in nucleic acid extracts, particularly those derived from plant matrices(Srividya *et al*., 2019) . LAMP's ability to be quickly adapted for point-of-care analysis makes it a viable tool for surveys or quarantine programmes that require quick, accurate, and specialised analysis.

2.9.1 Principals of LAMP primers design

The LAMP approach relies on auto cycling and high DNA strand displacement activity catalysed by Bst polymerase from *Geobacillus stearothermophilus* under isothermal conditions. A starting step and a step that combines a cycling amplification step with an elongation/recycling step make up the reaction(Varga & James, 2006). The isothermal amplification is carried out at 60–65 ◦C, the optimum temperature for Bst polymerase activity. A set of four primers that were able to recognise six different sequences within the target HBV viral DNA. The inner primers were made up of two different sequences that recognised the target DNA's sense and antisense sequences, whereas the outer primers recognised only one external sequence of the target DNA (Chander *et al*., 2014).

2.9.2 Mechanism of LAMP reaction:

2.9.2.1 Initial Step:

The first stage is carried out at 65 degrees Celsius, where primers can anneal to the specified sequence. The forward inner primer hybridises with the original reverse target sequence, and the 30-end flanked by the forward inner primer is used to start synthesis of the new forward strand. The forward outer primer then hybridises with the same original reverse target sequence, and the enzyme proceeds to synthesise this new forward strand until it reaches the 5' end of the first strand generated with the inner primer(Notomi *et al*., 2000).

The strand displacement of the initial forward strand formed using the forward inner primer happens as a result of the characteristics of the enzyme utilised in LAMP. Due to the complementarity of the reverse sequence from the inner primer to the target sequence, this split strand generates a self-hybridizing loop at one end. It also acts as a template for the

reverse inner and reverse outer primers, which, in a similar manner, cause the forward strand to be displaced, resulting in a dumbbell-like DNA structure (Varga & James, 2006).

2.9.2.2 Cycling Amplification and Elongation Step:

The forward inner primer hybridises to the loop of the strand generated in the previous stage and aids strand displacement, resulting in a new strand with an inverted copy of the target sequence in the stem region and a loop on the opposite side. Self-primed strand displacement DNA synthesis yields two products: one complementary strand and another with a doubly extended stem twice if the original and a loop on the opposite site. In the succeeding elongation and recycling stages, both strands are employed as templates for reverse-primed strand displacement synthesis. At each half of the cycle, the target sequence can be amplified three times(Martinelli *et al*., 2015). Because of the Bst DNA polymerase's strong displacement activity, a large amount of DNA with a high molecular weight is created quickly. In less than one hour, target DNA can be amplified to 109 copies. Finally, cauliflower-like structures with several loops and stem-loop DNAs of various lengths are formed(Mori *et al*., 2006) .

2.9.2.3 LAMP Acceleration:

Two extra primers, known as loop primers (loop forward (LF) and loop backward (LB), could be added to speed up the reaction. Apart from the loops hybridised by the inner primers and prime strand displacement, these primers hybridise with the stem-loops. Synthesis of DNA (Notomi, 2000). Currently, six primers are widely used in many diagnostic protocols to allow better specificity and sensitivity (Almasi, 2015) .

Another use of this technology was the development of plant pathology procedures using crude plant saps as a template, depending on the ability of the Bst polymerase to withstand different types of inhibitors. This application sped up the diagnosis process by obviating the need for any prior sample preparation or DNA or RNA purification (Selvaraj *et al*., 2019).

As a result, the diagnosis time was reduced by 2–3 hours, which was required to complete the target organism's DNA/RNA extraction. At the same time, the LAMP reaction costs are significantly lowered because no expensive nucleic acid extraction kits are required.

2.9.3 General and Specific Considerations for Primers Design

The design of primers and/or probes is one of the most important elements impacting the success and quality of nucleic acid amplification-based procedures (Kamel, 2003). The LAMP primers require eight distinct sections of the target nucleic acid sequence to be chosen. The development of dimers among primers must be avoided at all costs, especially for FIP and BIP primers, which are typically 40 nucleotides long. The F2 sequence (at its 30 end) is complementary to the F2c area, while the FIP sequence (at its 50 ends) is the same as the F1c region. The B2 sequence (at its 30 end) is complementary to the B2c region, while the B1c sequence (at its 50 end) is the same as the B1c region. The complementary strand corresponding to the region between F1 and F2 is used to construct LF primers, while the complementary strand corresponding to the region between B1 and B2 is used to design LB primers (Notomi *et al*., 2000).

2.9.5 Visualisation of LAMP amplification products:

During the continuous amplification of a LAMP reaction under isothermal conditions, a substantial amount of target DNA, as well as a considerable amount of by-product, is produced. Positive LAMP reactions can be detected using a variety of approaches.

There are two types of visualisation outcome approaches: one that is based on reading results at the conclusion of the amplification process (end point-based methods) and another that is based on reading results throughout the process (during point-based methods) (real-time-based methods). Fluorescent molecules, such as ethidium bromide (EtBr) or SYBR Green I (Iwamoto *et al*., 2003).

By using post-amplification dyes in the solution or by using gel electrophoresis, LAMP DNA amplicons may be seen. These techniques necessitate opening the tubes, and because so much DNA is generated during the reaction, there is a possibility of contamination(Fischbach *et al*., 2015; Karthik *et al*., 2014) (Karthik *et al*., 2014; Fischbach *et al*., 2015). There are a number of visualisation techniques that may be employed for closed-tube amplification processes to solve that issue.

By measuring the change in metal ion concentration in the solution during LAMP, calcein and HNB can distinguish enhanced products. In this process, pyrophosphate ions are created in large quantities and combine with metal ions, such manganese, to form insoluble salts. Due to the quenching of the reaction, the manganese content drops, turning the

solution orange due to the manganese ions that had previously coupled with calcein for the reaction. Once LAMP begins in the presence of DNA, the newly formed pyrophosphate ion depletes calcein of manganese ions, allowing calcein to react with leftover magnesium to produce fluorescence (Tanner *et al*., 2015). LAMP products can also be found using a photometer for quantitative detection or by visually observing the turbidity of the solution. (Duan *et al*., 2014; Goto *et al*., 2009).

2.9.7 LAMP Application in Plant Virology:

Viruses are the most common cause of plant disease in field crops, fruit trees, vegetables, and industrial plants. Plant viruses are known to cause disease in over 900 different species. Many of them have a diverse set of hosts. (Abramovitch & Martin, 2004).

Fukuta and colleagues published the first LAMP procedures in phytopathology in 2003 for the detection of two plant viruses on horticultural crops, tomato yellow leaf curl virus (TYLCV; Circ-ssDNA+/) and tomato yellow leaf curl virus (TYLCV; Circ-ssDNA+/)(Fukuta *et al*., 2003) and Japanese yam mosaic virus (JYMV; ssRNA+)(Peng *et al*., 2012). Following that, alternative methods for various plant viruses and viroids, including economically and agronomically relevant agents, were devised.

BSV, a pathogenic virus on bananas, was also identified using LAMP with a sensitivity of 1 pg DNA template, which was 100 times higher than PCR for the same sample. They also created a LAMP test that can identify mosaic virus on wheat with a sensitivity of 100 times higher than PCR (Peng *et al*., 2012). The combination of LAMP with a reverse transcriptase enzyme, known as RT-LAMP, has also been frequently used to detect retrovirus infections. For example, RT-LAMP delivers high accuracy and specificity, as well as sensitivity between 100 and 1000 times larger than RT-PCR for diagnosing blight disease on soybean seed caused by Bean pod mottle virus(Wei *et al*., 2012).

Turnip mosaic virus, the pathogen of at least 318 species in 156 genera and 43 families, predominantly cruciferous plants, has been detected using an excellent RT-LAMP test (Varga & James, 2006).

The introduction of LAMP portable equipment has recently enabled ‘real-time' pathogen detection on-field, enabling their diagnosis during routine surveys, sanitary selection, and eradication operations (Wilisiani *et al*., 2019)

3 MATERIAL AND METHODS

The present study was conducted in the department of Botany, Jamia Hamdard University, New Delhi. This chapter describes the materials and methods used during the course of the investigation.

3.1 Glassware Used:

Conical Flask, Measuring Cylinder, Mortal pestle, Centrifuge tubes

3.2 Equipment Used:

Autoclave: it provides a physical method for disinfection and sterilization. They work with a combination of steam, pressure and time.

NanoDrop UV Visible Spectrophotometers: it enables the rapid and simple quantification and evaluation of sample purity, such as that of proteins and nucleic acids. Due to their quickness, ease of use, and reliable performance, NanoDrop devices are practically indispensable for every lab that works with biological material.

Thermocycler: They are used in molecular biology for a variety of procedures, including DNA sequencing, cloning, probe production, DNA and RNA quantification, pattern analysis of gene expression, and the detection of sequence-tagged sites. When highly precise temperature control is needed, for as when heating tissue slides for in situ hybridization, a thermocycler can be employed. The study of temperature-dependent kinetics is made possible by the controlled temperature ramping given by a thermocycler.

Water bath: In the lab, samples are heated using a water bath. To keep liquid samples in test tubes, cups, or other containers at a consistent temperature in water medium, water baths are used to heat or cool the samples. They are perfect for items like flammable liquids or biological materials that cannot be subjected to direct heat from an incubator, oven, or hotplate. They are employed in many different types of laboratories.

Gel Doc: Gel documentation, also known as gel imaging, devices are used to record and measure tagged nucleic acid and protein in a variety of media, including agarose, acrylamide, and cellulose. Systems are available in a range of configurations based on throughput and sample type.

Centrifuge: A centrifuge is a laboratory apparatus used to separate fluids, either gas or liquid, depending on density. Separation is accomplished by rapidly rotating a vessel holding material; centrifugal force forces heavier elements to the exterior of the vessel. There are several different types of centrifuges accessible to researchers, ranging from enormous floor models to micro-centrifuges.

Vortexer: Vortex mixers are a common method for mixing laboratory substances in test tubes, well plates, or flasks. They employ a very simple mechanism to precisely agitate materials and induce reactions or homogenization.

Weighing balance: Analytical balances are very sensitive laboratory devices used to quantify mass precisely. Analytical balances contain a draught shield or weighing chamber to keep air currents from affecting the extremely tiny samples.

3.3 DIAGNOSIS OF DISEASE BY RT-PCR:

Plant material used to isolate CaCV:

Tomato fruits having typical tospovirus infection were gathered from Local Market, Okhla Mandi, New Delhi-110025

Total RNA Isolation:

Materials and Reagents required-

- Autoclaved Mortar Pistil
- Autoclaved Tips
- CTAB buffer (2%), prepared as given in table1

Table 1 - CTAB buffer components

S. No.	CTAB buffer components	For 100 ml
1.	CTAB	2g
2.	PVP (3%)	3g
3.	NaCl (5M)	40ml
4.	Tris- HCl (1M, pH-8)	2.5 ml
5.	EDTA (0.5M, pH- 8)	1.25 ml
6.	β- M.E. (1-2%)	10-20µl

- β- Mercaptoethanol
- Chloroform
- Isoamyl Alcohol
- LiCl (7.5 mM)
- 70% Ethanol
- RNAse free water

6 tomato plant samples showing symptoms of tospovirus were taken and are labelled as TS1, TS2, TS3, TS4, TS5, TS6.

Procedure –

- Take 0.1~0.2 gm of tomato pericarp from each sample and grind it in pestle with mortar
- Transfer the grinded powder to 2 ml Eppendorf tubes and add prewarmed (65ºC) 1 ml 2% CTAB buffer to it.
- Add 10µl β- Mercaptoethanol to it followed by 1-minute vertexing and 10 minutes incubation at 65 ºC.
- Centrifuge at 13000 rpm for 2 minutes
- Take the supernatant to other 2 ml Eppendorf and add equal volume of Chloroform; isoamyl alcohol (24:1) followed by 1-minute vertexing.
- Centrifuge it at 13000 for 10 mins
- Take the supernatant obtained carefully and add half the amount of LiCl.
- Store at -20 ºC for 10 mins
- Centrifuge at 13000 rpm for 30 minutes.
- Decant the supernatant.
- Wash with 80% ethanol (twice)
- Let the pellets airdry and then add 50µl RNase free water for dilution.
- Prepare 1% Agarose gel and load 1 µl Loading Dye and 5 µl sample for visualization of RNA using Gel Dock.
- Quantification of RNA has been done using Nanodrop and is mentioned in table 8

cDNA Synthesis

- cDNA was synthesized using a reverse transcriptase enzyme- MMLV-RT enzyme in a 20 µl reaction mixture containing 500 ng of total RNA isolated from the infected and non-infected samples of tomato.
- The following components were added to prepare reaction mixture in the order mentioned in (table 2).

Table 2 - Aliquot Preparation for cDNA synthesis

Components	Concentration	Volume(µl)
5X- RT-buffer (Thermo)	-	04.0 µl
dNTPs	40mM	01.0 µl
Oligo-dT and hexamer mix	10 mM	01.0 µl
RNA	500 ng	10.00 µl
Water	-	03.50 µl
MMLV-RT enzyme	100 unit	0.5 µl
	Total volume	20 µl

Protocol:

- Place 10 µl of each RNA sample in micro-centrifuge tubes (MCTs), centrifuge briefly in a microcentrifuge and then incubate it at 72 C for 5 mins and then transfer it rapidly to ice.

Prepare a 20 µl aliquot reaction with the help of following components

- Place it in a thermocycler at following conditions given in table 3.

Table 3 - Temperature conditions for cDNA synthesis

Temperature	Time
25 ºC	10 minutes
42 ºC	60 minutes
80 ºC	05 minutes

- Store the c-DNA at -20 C until use.

Polymerase Chain Reaction –

- Mix the reaction carefully in microcentrifuge tubes making up to the volume to 20 µl (table 4&5). If required, quickly spin the tube to force aliquot bottom.

Table 4 - PCR Aliquot (Volume makeup to 20µl)

Components	**Volume**
cDNA	2.00 µl
Forward Primer – TOS-F	0.50 µl
Reverse Primer – TOS-R	0.50 µl
10X rxn buffer	2.00 µl
dNTP's	0.50 µl
Taq. Pol. (Genei)	0.25 µl
Water	14.25 µl

- Transfer the tubes to PCR machine (Bio-Rad).

Table 5 - Thermocycling conditions for PCR

Step	Temperature	Time
Initial Denaturation	95 ºC	2 minutes
35 cycles	94 ºC 58 ºC 72 ºC	30 seconds 40 seconds 45 seconds
Final Extension	72 ºC	7 minutes
Hold	04 ºC	∞

- Store the PCR product at -20 C till further use

Gel electrophoresis:

Materials and reagents

Table 6 - Chemicals and tools required for Agarose Gel Electrophoresis

1.	**For Preparation**	Tank, Tray, Comb
		Autoclaved distilled water
		Agarose
		1X TAE buffer
		Microwave
		Conical flask
		Measuring cylinder
		EtBr
2.	**For Loading**	Pipette
		Pipette tips -20 µl (autoclaved)
		Gel Loading Dye
3.	**For Visualization**	Gel Documentation system

Gel Preparation for RNA –

- Prepare 100 ml TAE buffer using 50 X TAE (stock) by diluting 2 ml 50X TAE in 98 ml autoclaved distilled water
- Clean the tray and comb well using ethanol and fix the mold
- Place the comb such that complete wells were formed when agarose is added.
- Prepare 1% agarose gel as given in (table 6).
- Weigh 0.2 gm of agarose powder and mix it gently in 20 / 40 ml (depending on single or double comb gel) 1X TAE buffer by providing heat using microwave to avoid any bubbles.
- Leave it to cool down to ~45-50 C and then add the staining dye say EtBr
- Pour the solution into the tray and let it solidify for nearly 20-30 minutes.
- After that remove the comb carefully and position the gel in gel electrophoresis chamber.

Loading:

- Mix the RNA samples with gel loading dye using pipettes Table 6:

Table 7 - Composition of RNA loading Samples

RNA sample	5 µl
Gel Loading Dye (Thermo Scientific 6X DNA Loading Dye)	1 µl

- Load the mixture slowing into the wells, avoiding bubbles as mentioned in table 7
- Attach the electrical leads so the RNA can move towards the anode.
- Apply voltage
- Run the gel until the gel loading dye has moved an appropriate distance.

3.4 Sequence of PCR products and phylogenetic analysis:

The sequence of the virus isolate under this research was compared with the sequences of selected Tospovirus species obtained from the NCBI gene bank database. Then, the sequence was uploaded for Multiple sequence alignment using multalin (http://multalin.toulouse.inra.fr/multalin) followed by phylogenetic analysis using MEGA 6.0 software (www.megasoftware.net) generated using Neighbour joining tree method, bootstrapped with 24-25 replicates.

4 RESULT AND DISCUSSION

4.1 Symptomatology:

Tomato fruits showing typical symptoms of tospovirus were collected from Okhala Mandi, New Delhi. Chlorotic ring marks were clearly visible on the red ripe tomato fruits (Fig.1).

Figure 1- Chlorotic spots on Tomato fruit samples

4.2 RNA isolation

Total RNA was isolated from the pericarp of six tomato fruits by CTAB methods. The integrity of the isolated RNA was checked by running it on 1% agarose gel and visualized under Gel Dock system after stanning with EtBR (Fig 2&3).

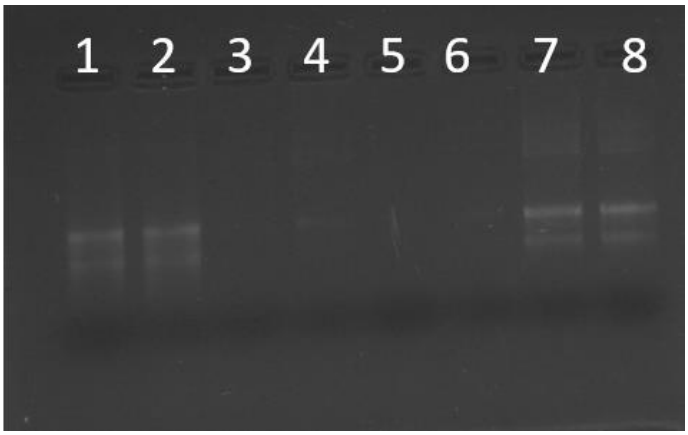


Figure 2- - Bands of 18S and 28S RNA were visible for TS1 in lane 1 and 2 & TS4 in lane 7 and 8

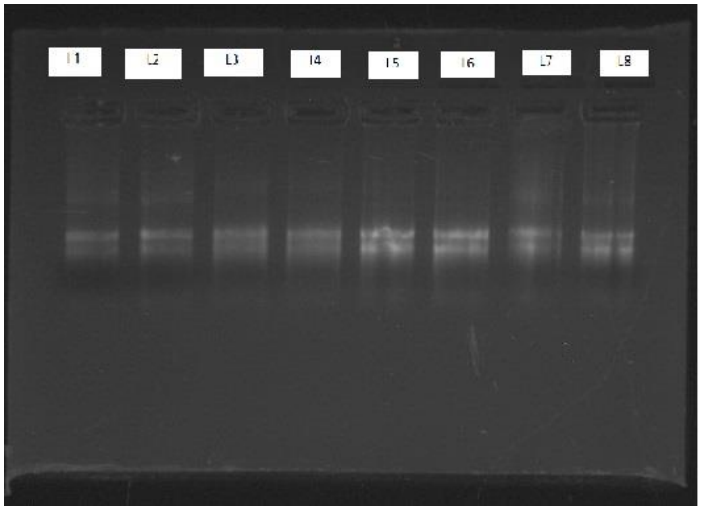


Figure 3- Bands of 18S and 28S RNA were visible for TS2 in Lane 1 & 2, TS3 in Lane 3 & 4, TS5 in Lane 5 & 6 and TS6 in Lane 7 & 8

4.3 Quantification of RNA-

Table 8: Quantification of RNA

S. No.	Sample	Concentration		Ratio of Absorbance (RoA)
1.	TS1	51.5 ng/µl		2.16
2.	TS2	50.5 ng/µl		2.29
3.	TS3	78.6 ng/µl	**A260/A280**	2.15
4.	TS4	33.3 ng/µl		2.25
5.	TS5	150 ng/µl		2.14

6.	TS6	106 ng/µl		2.16

4.4 RT PCR based detection of Tospovirus

A DNA fragment of ~840 bp corresponding Tospovirus-NP gene was amplified from six tomato fruit samples by RT-PCR using degenerate primers (TosF/R) (Fig: 4 & 5).

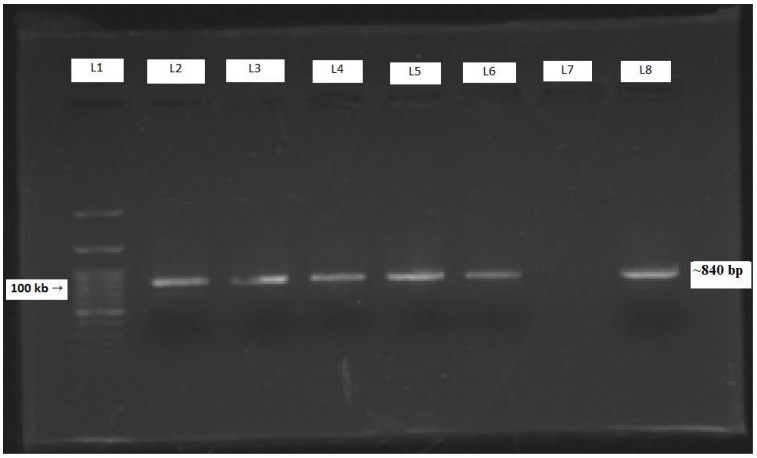


Figure 4- Visualization of PCR product by using the c-DNA synthesized with the help of MMLV-RT Enzyme (Lane 1- DNA 100 bp ladder, Lane 2- TS1, Lane 3

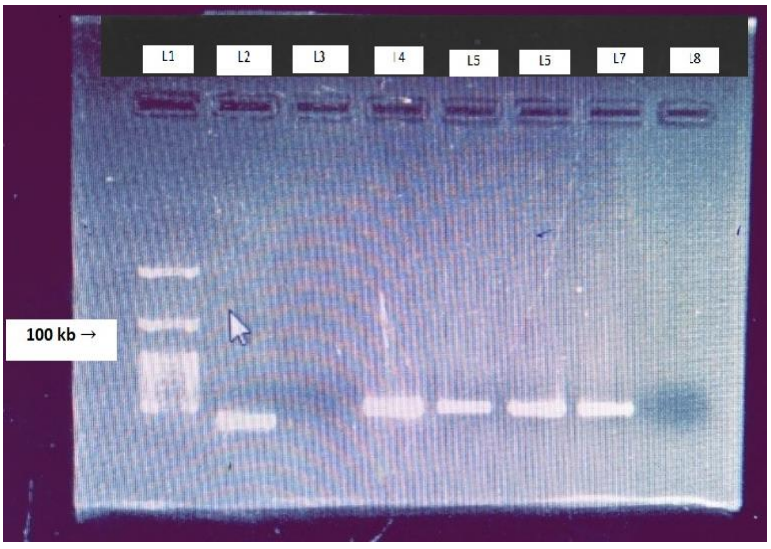


Figure 5 - Interpretation of Bands corresponding 840bp Tospovirus NP gene

(Lane 1- 100kb ladder, Lane 2 -TS6, Lane 3- Blank, Lane 4 – TS1, Lane 5- TS2, Lane 6- TS3, Lane7- TS4, Lane8- TS5)

4.5 Sequencing of PCR product

One of the PCR amplicons obtained from the infected tomato fruit was sequenced by sanger sequencing by using TosR (reverse primer)

>0622_839_004_PCR_P2_R_B05.ab1

CAGAGTAATTAGGCACTTAAATGGCTTGGGGGCCATATATTTAACTTGCTCATCATACTT
CTTAAGTGAGATGGAACTAGCAGTACCAGGGTTGCTTTCACTAAGCAACTTGACAGCCTG
TTTGAACAGTGTGTTCAGATCTTCCTTAAATTCTATTTGAGAAGCAGAAAGAACTTTAGC
CACTTTACAAACCTGTTCATAGGTAGAGAAGTTTTTGATGCCCAATTTCTCTTTTTTAAC
ATTTTGGTAATAAGCTAGTGGGAAAATAATTGGTGCAAGGCCTTTAATACTGGATAATAG
TGGAAGAGGACCACCAATACATAACATCATCCTGAGAGCACATGAATCAAACAAAGCAGG
TATGTTCAATCCATATGCTGCCACCAAAGGTAATTCCATTACCTTTGCATACATCTCTTG
TTTAGCAGCTTCATTTTTATTCTTCTCTACCATATTGATCATCTTAACTCTTATCAAAGC
TTCTGTTCTCTTAAAAGTCCAATCCTCTGGACCAACATCAGCATCCGTAGAAACAATTGT
TTTTTCACAGAACTTATACTTTCCACTTTTGCAAGCAGCAAAAATCTGCTTTCTACACTT
CAAGATATTAAGACAGTTTGTGAATGTCATTTCAATGCCTTTGTTATTATCATAGAATGT
CTTGAAACTGAATCCAGGAGTTGAATCCTCAGTTTCAATTTCAACATCTGCAGTTCCACC
AGCCAAGAGGTCTCTAATTTTCTTGTCGAAAAGTTGCCTAACGTTAAGAAAAAAAATAAA
AAGAGAAAGAAAACCTTCTCTGGAATTAGAAAA

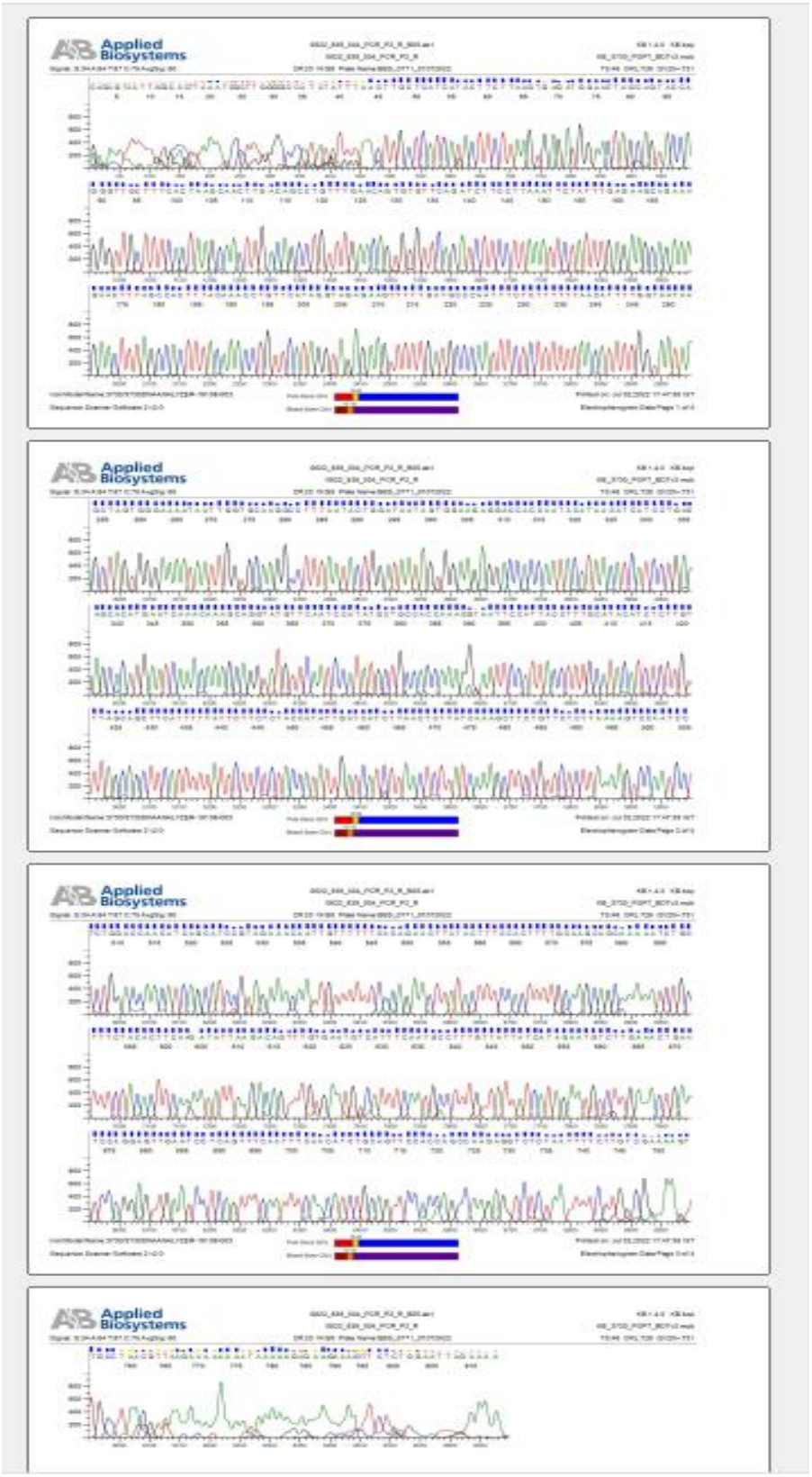


Figure 6: Chromatogram of obtained sequence

4.6 BLAST analysis of sequence

BLASTn analysis (NCBI) of the sample sequence obtained showed 96-99% similarity with Capsicum Chlorosis Virus at nucleotide level(Fig.6)

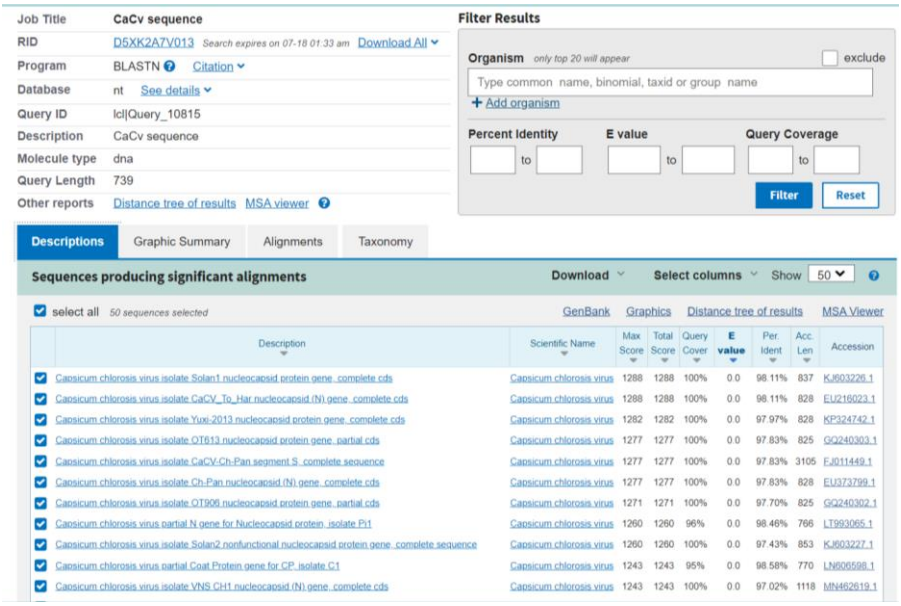


Figure 7: BLASTn result for the sample sequence

BLASTp analysis (NCBI) of sample sequence showed 95-99% similarity with CaCV at amino acid level (Fig.7)

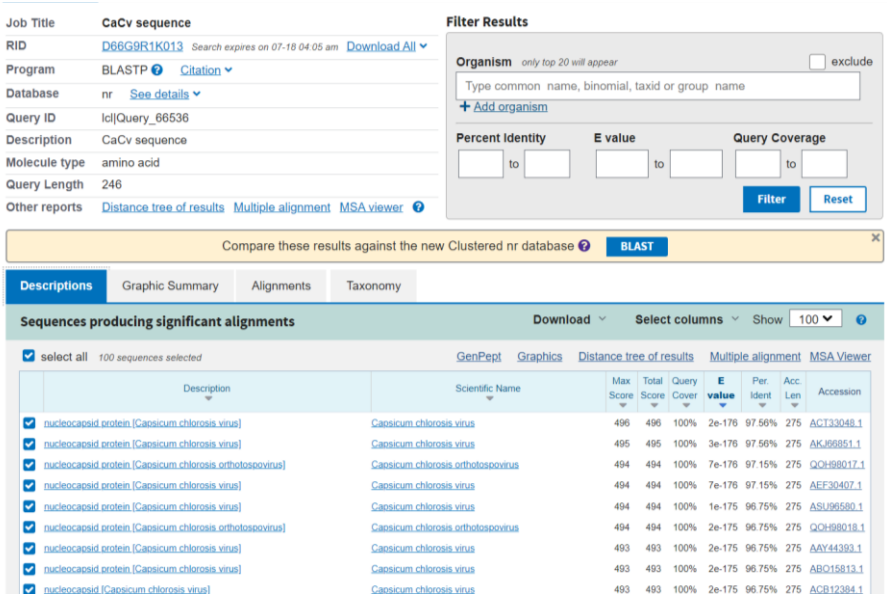
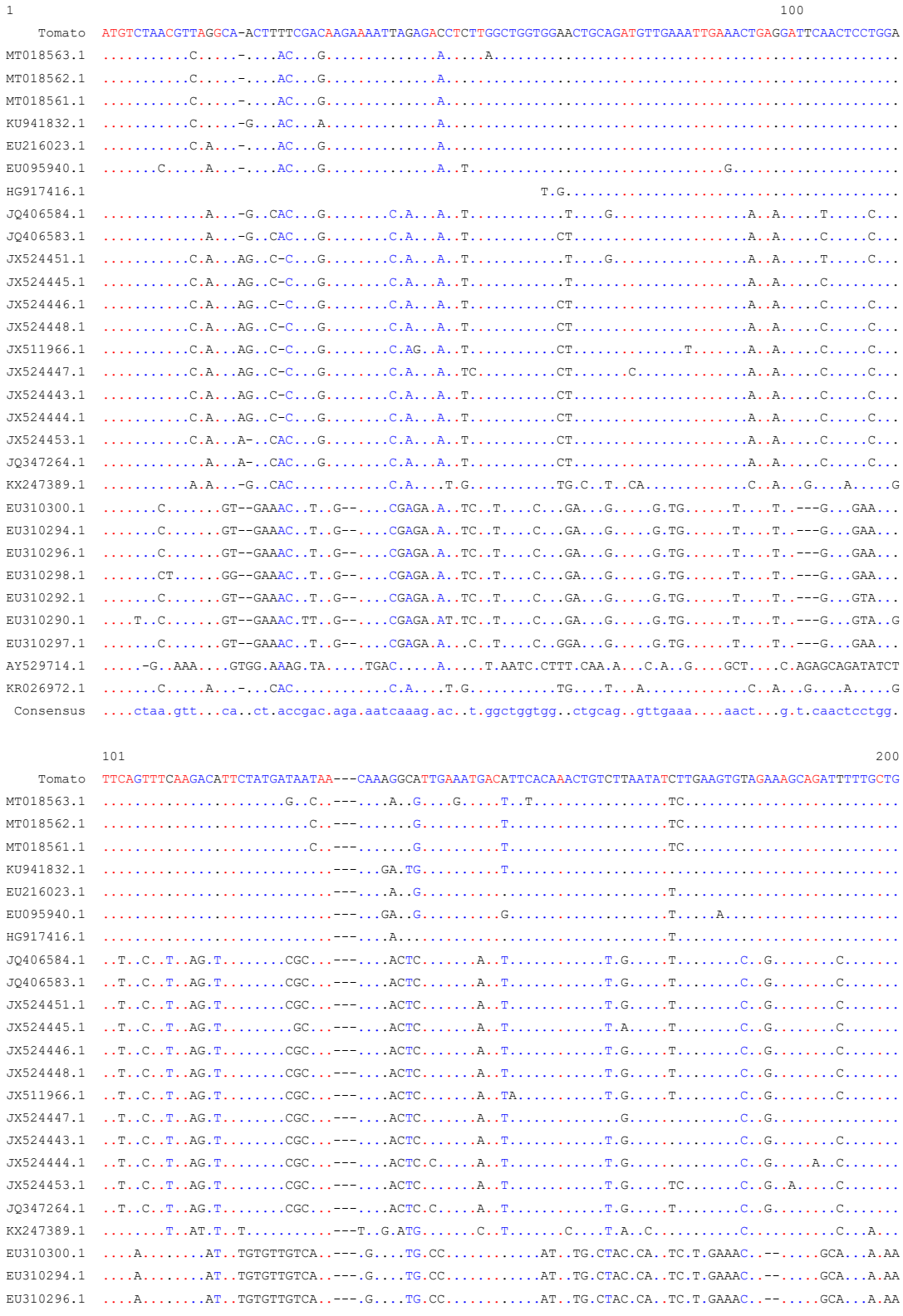
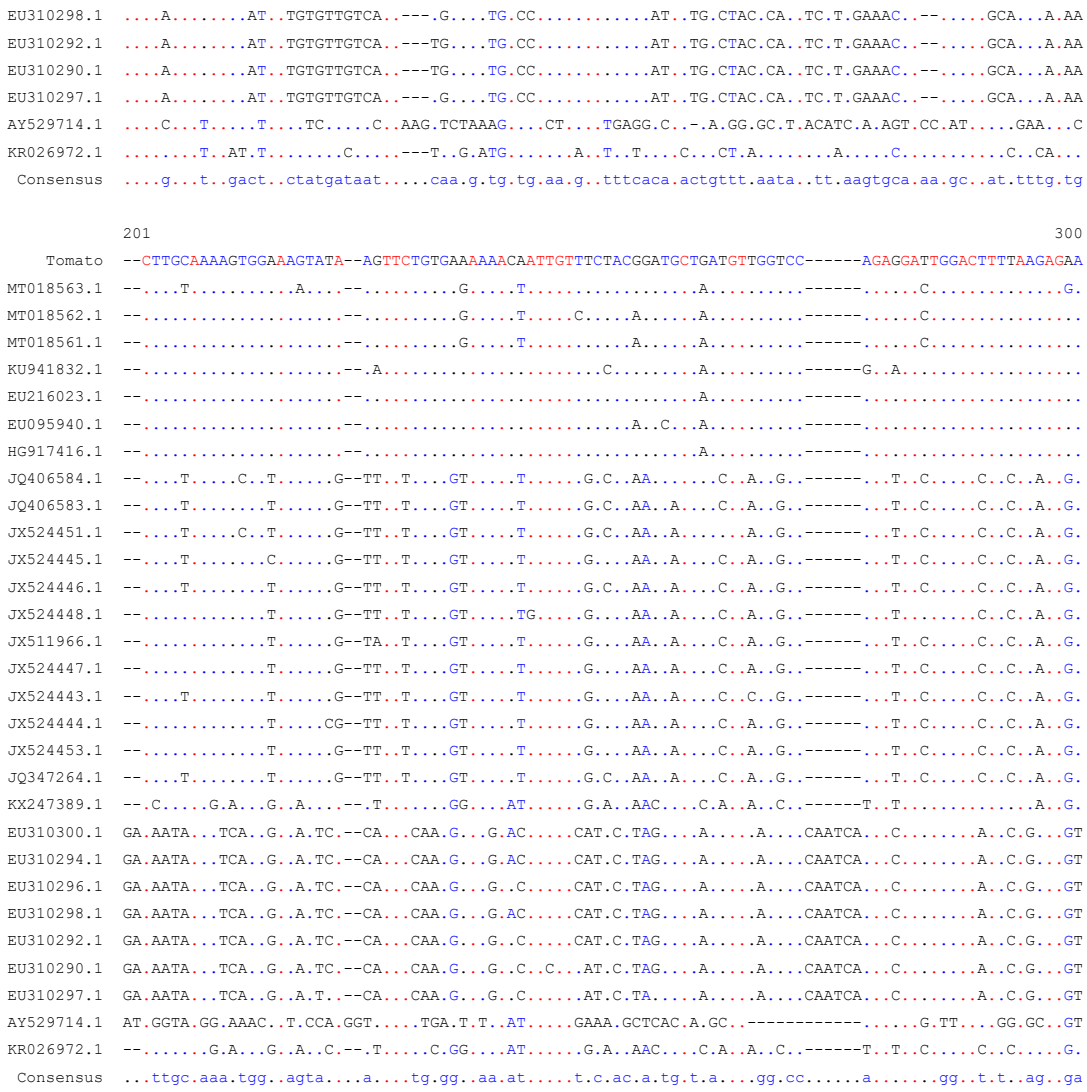


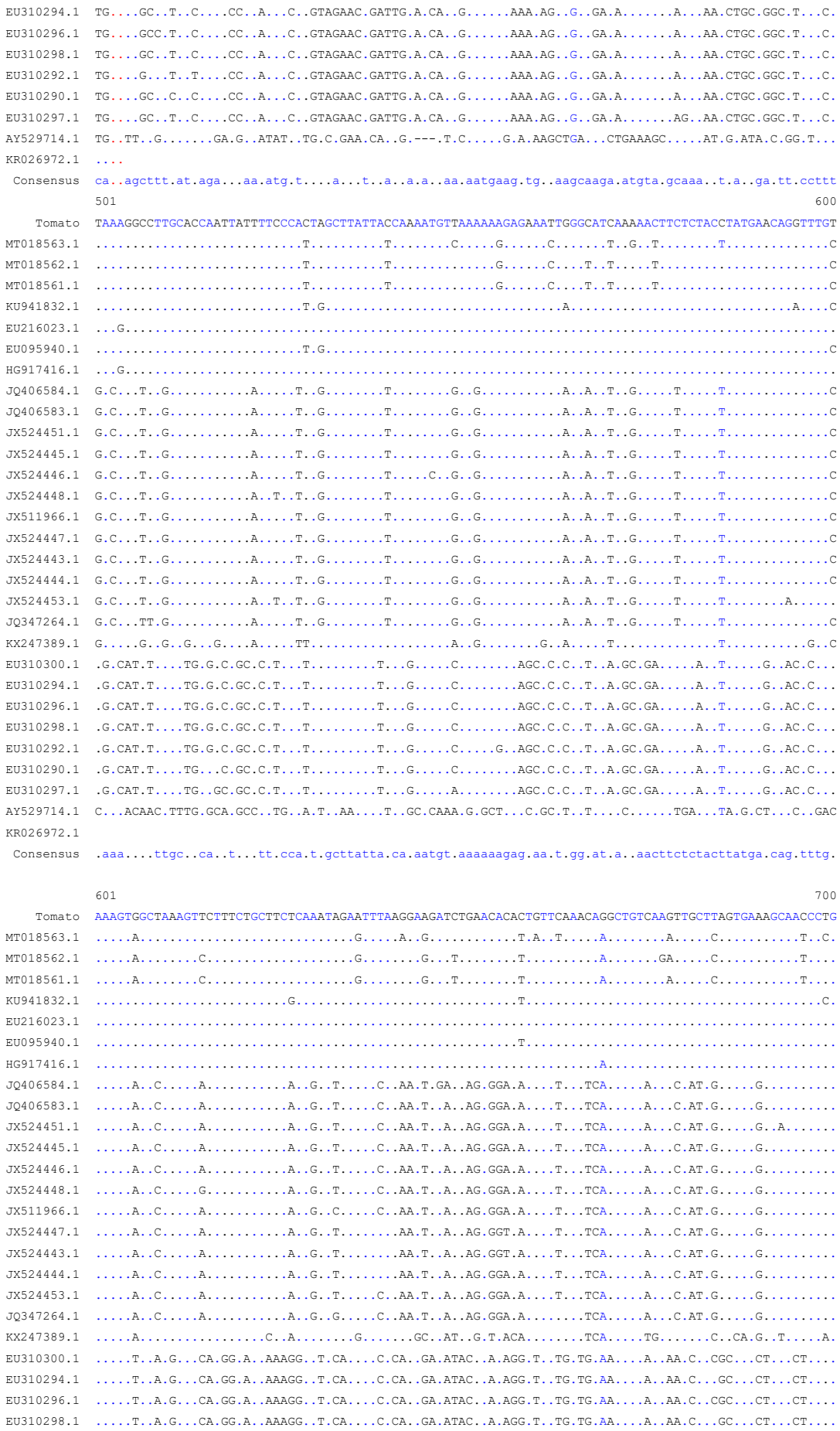
Figure 8: BLASTp Analysis for the sample sequence

4.7 Multiple sequence alignment

For understanding the variation between the sequence obtained and the other isolates of CaCV reported from all over India, multiple sequence alignment was done using an online alignment tool named multalin (http://multalin.toulouse.inra.fr/multalin/).




EU310298.1 ....A........AT..TGTGTTGTCA..---.G....TG.CC.............AT..TG.CTAC.CA..TC.T.GAAAC..--.....GCA...A.AA
EU310292.1 ....A........AT..TGTGTTGTCA..---TG....TG.CC.............AT..TG.CTAC.CA..TC.T.GAAAC..--.....GCA...A.AA
EU310290.1 ....A........AT..TGTGTTGTCA..---TG....TG.CC.............AT..TG.CTAC.CA..TC.T.GAAAC..--.....GCA...A.AA
EU310297.1 ....A........AT..TGTGTTGTCA..---.G....TG.CC.............AT..TG.CTAC.CA..TC.T.GAAAC..--.....GCA...A.AA
AY529714.1 ....C...T.....T....TC.....C..AAG.TCTAAAG....CT....TGAGG.C..-.A.GG.GC.T.ACATC.A.AGT.CC.AT.....GAA...C
KR026972.1 ........T..AT.T........C.....---T..G.ATG.......A..T..T....C...CT.A........A.....C...........C..CA...
Consensus ....g...t..gact..ctatgataat.....caa.g.tg.tg.aa.g..tttcaca.actgttt.aata..tt.aagtgca.aa.gc..at.tttg.tg
201
300
Tomato --CTTGCAAAAGTGGAAAGTATA--AGTTCTGTGAAAAAACAATTGTTTCTACGGATGCTGATGTTGGTCC------AGAGGATTGGACTTTTAAGAGAA
MT018563.1 --....T...........A....--..........G.....T..................A..........------......C..............G.
MT018562.1 --.......................--..........G.....T.....C.....A......A..........------......C..............
MT018561.1 --.......................--..........G.....T...........A......A..........------......C..............
KU941832.1 --.......................--.A.........................C.........A..........------G..A..................
EU216023.1 --.......................--.....................................A..........------.....................
EU095940.1 --.......................--.............................A..C...A..........------.....................
HG917416.1 --.......................--.....................................A..........------.....................
JQ406584.1 --....T.....C..T......G--TT..T....GT.....T......G.C..AA.......C..A..G..------...T..C.....C..C..A..G.
JQ406583.1 --....T.........T......G--TT..T....GT.....T......G.C..AA..A....C..A..G..------...T..C.....C..C..A..G.
JX524451.1 --....T.....C..T......G--TT..T....GT.....T......G.C..AA..A.......A..G..------...T..C.....C..C..A..G.
JX524445.1 --....T.........C......G--TT..T....GT.....T......G....AA..A....C..A..G..------...T..C.....C..C..A..G.
JX524446.1 --....T.........T......G--TT..T....GT.....T......G.C..AA..A....C..A..G..------...T..C.....C..C..A..G.
JX524448.1 --.............T......G--TT..T....GT.....TG.....G....AA..A....C..A..G..------...T........C..C..A..G.
JX511966.1 --.............T......G--TA..T....GT.....T......G....AA..A....C..A..G..------...T..C.....C..C..A..G.
JX524447.1 --.............T......G--TT..T....GT.....T......G....AA..A....C..A..G..------...T..C.....C..C..A..G.
JX524443.1 --....T.........T......G--TT..T....GT.....T......G....AA..A....C..C..G..------...T..C.....C..C..A..G.
JX524444.1 --.............T.....CG--TT..T....GT.....T......G....AA..A....C..A..G..------...T..C.....C..C..A..G.
JX524453.1 --.............T......G--TT..T....GT.....T......G....AA..A....C..A..G..------...T..C.....C..C..A..G.
JQ347264.1 --....T.........T......G--TT..T....GT.....T......G.C..AA..A....C..A..G..------...T..C.....C..C..A..G.
KX247389.1 --.C.....G.A...G..A....--.T.......GG....AT......G.A..AAC....C.A..A..C..------T..T..............A..G.
EU310300.1 GA.AATA...TCA..G..A.TC.--CA...CAA.G...G.AC.....CAT.C.TAG....A.....A....CAATCA...C........A..C.G...GT
EU310294.1 GA.AATA...TCA..G..A.TC.--CA...CAA.G...G.AC.....CAT.C.TAG....A.....A....CAATCA...C........A..C.G...GT
EU310296.1 GA.AATA...TCA..G..A.TC.--CA...CAA.G...G..C.....CAT.C.TAG....A.....A....CAATCA...C........A..C.G...GT
EU310298.1 GA.AATA...TCA..G..A.TC.--CA...CAA.G...G.AC.....CAT.C.TAG....A.....A....CAATCA...C........A..C.G...GT
EU310292.1 GA.AATA...TCA..G..A.TC.--CA...CAA.G...G..C.....CAT.C.TAG....A.....A....CAATCA...C........A..C.G...GT
EU310290.1 GA.AATA...TCA..G..A.TC.--CA...CAA.G...G..C..C...AT.C.TAG....A.....A....CAATCA...C........A..C.G...GT
EU310297.1 GA.AATA...TCA..G..A.T..--CA...CAA.G...G..C......AT.C.TA.....A.....A....CAATCA...C........A..C.G...GT
AY529714.1 AT.GGTA.GG.AAAC..T.CCA.GGT.....TGA.T.T..AT.....GAAA.GCTCAC.A.GC..------------......G.TT....GG.GC..GT
KR026972.1 --.......G.A...G..A..C.--.T.....C.GG....AT......G.A..AAC....C.A..A..C..------T..T..C.....C..C.....G.
Consensus ...ttgc.aaa.tgg..agta....a....tg.gg..aa.at.....t.c.ac.a.tg.t.a....gg.cc......a.......gg..t.t..ag..ga
301
400
Tomato CAGAAGCTTTGATAAGAGTTAAGATGATCAATATGGTAGAGAAGAATAAAAATGAAGCTGCTAAACAAGAGATGTATGCAAAGGTAATGGAATTACCTTT
MT018563.1 ....A....GC..........T...C.G.....................G...........AC.
MT018562.1 ....A..A.GC..G.......T...C.....................G...........A..
MT018561.1 ....A..A.GC..G.......T...C.....................G...........A..
KU941832.1 ..............................C.........A....G...T.......G.....G.....................................
EU216023.1 ....C...
EU095940.1 ..........A.....................C.........A....G...G......AG.....GG.....................G.............
HG917416.1 ....C...
JQ406584.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T.....C..G..G........CAAT..AA.............A..
JQ406583.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T.....C..G..G........CAAT..AA.............A..
JX524451.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T.....C..G..G........CAAT..AA.............A..
JX524445.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T.....C..G..G........CAAT..AA.............A..
JX524446.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T.....C..G..G........CAAT..AA.............A..
JX524448.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T........G.......C...CAAT..AA.............A..
JX511966.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T........G...........CAAC..AA.............A..
JX524447.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T........G..G........CAAT..AA.............A..
JX524443.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T........G..G........CAAT..AA.C...........A..
JX524444.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T........G..G........CAAT..AA.............A..
JX524453.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T........G..G........CAAT..AA.............A..
JQ347264.1 ..........C..C..GACC..A...GCT.G......T..A....GC..G.....T.....C..G..G........CAAT..AA.............A..
KX247389.1 ..........T..C...ACA..A...G.T.G......G.....A.G..CT......AA...C..G...........CA.G..AA........C.G..A..
EU310300.1 TG....GC..T..C....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......A...AA.CTGC.GGC.T...C.



EU310294.1 TG....GC..T..C....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......A...AA.CTGC.GGC.T...C.
EU310296.1 TG....GCC.T..C....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......A...AA.CTGC.GGC.T...C.
EU310298.1 TG....GC..T..C....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......A...AA.CTGC.GGC.T...C.
EU310292.1 TG....G...T..T....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......A...AA.CTGC.GGC.T...C.
EU310290.1 TG....GC..C..C....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......A...AA.CTGC.GGC.T...C.
EU310297.1 TG....GC..T..C....CC..A...C..GTAGAAC.GATTG.A.CA..G......AAA.AG..G..GA.A.......AG..AA.CTGC.GGC.T...C.
AY529714.1 TG..TT..G.......GA.G..ATAT..TG.C.GAA.CA..G.---.T.C.....G.A.AAGCTGA...CTGAAAGC.....AT.G.ATA.C.GG.T...
KR026972.1 ....
Consensus ca..agcttt.at.aga...aa.atg.t....a...t..a..a.a..aa.aatgaag.tg..aagcaaga.atgta.gcaaa..t.a..ga.tt.ccttt

501 600

Tomato TAAAGGCCTTGCACCAATTATTTTCCCACTAGCTTATTACCAAAATGTTAAAAAAGAGAAATTGGGCATCAAAAACTTCTCTACCTATGAACAGGTTTGT
MT018563.1 .....T.........T........C.....G......C.......T..G..T........T.............C
MT018562.1 .....T.........T.............G......C....T..T.....T.....................C
MT018561.1 .....T.........T.............G......C....T..T.....T.....................C
KU941832.1 .....T.G..............................A.............................A....C
EU216023.1 ...G.....
EU095940.1 .....T.G.....
HG917416.1 ...G.....
JQ406584.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JQ406583.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524451.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524445.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524446.1 G.C...T..G..........A.....T..G........T.....C..G..G..........A..A..T..G.....T.....T.............C
JX524448.1 G.C...T..G..........A..T..T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX511966.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524447.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524443.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524444.1 G.C...T..G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
JX524453.1 G.C...T..G..........A..T..T..G........T........G..G..........A..A..T..G.....T.....T........A......
JQ347264.1 G.C...TT.G..........A.....T..G........T........G..G..........A..A..T..G.....T.....T.............C
KX247389.1 G.....G..G..G...G....A.....TT...................A..G........G..A.....T.............T...........G..C
EU310300.1 .G.CAT.T....TG.G.C.GC.C.T...T.........T...G.....C........AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
EU310294.1 .G.CAT.T....TG.G.C.GC.C.T...T.........T...G.....C........AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
EU310296.1 .G.CAT.T....TG.G.C.GC.C.T...T.........T...G.....C........AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
EU310298.1 .G.CAT.T....TG.G.C.GC.C.T...T.........T...G.....C........AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
EU310292.1 .G.CAT.T....TG.G.C.GC.C.T...T.........T...G.....C.....G..AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
EU310290.1 .G.CAT.T....TG...C.GC.C.T...T.........T...G.....C........AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
EU310297.1 .G.CAT.T....TG..GC.GC.C.T...T.........T...G.....A........AGC.C.C..T..A.GC.GA.....A..T.....G..AC.C...
AY529714.1 C...ACAAC.TTTG.GCA.GCC..TG..A.T..AA....T..GC.CAAA.G.GCT...C.GC.T..T....C......TGA...TA.G.CT...C..GAC
KR026972.1
Consensus .aaa....ttgc..ca..t...tt.cca.t.gcttatta.ca.aatgt.aaaaaagag.aa.t.gg.at.a..aacttctctacttatga.cag.tttg.

601 700

Tomato AAAGTGGCTAAAGTTCTTTCTGCTTCTCAAATAGAATTTAAGGAAGATCTGAACACACTGTTCAAACAGGCTGTCAAGTTGCTTAGTGAAAGCAACCCTG
MT018563.1 .....A.....G.....A..G.....T.A..T.....A........A.....C.....T..C.
MT018562.1 .....A.....C.....G.....G...T.....T.....A.....GA.....C.....T....
MT018561.1 .....A.....C.....G.....G...T.....T.....A.....A.....C.....T....
KU941832.1 .....G.....T.....C.
EU216023.1 .....
EU095940.1 .....T.....
HG917416.1 .....A.....
JQ406584.1 .....A..C.....A.....A..G..T.....C..AA.T.GA..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JQ406583.1 .....A..C.....A.....A..G..T.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JX524451.1 .....A..C.....A.....A..G..T.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G..A.....
JX524445.1 .....A..C.....A.....A..G..T.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JX524446.1 .....A..C.....A.....A..G..T.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JX524448.1 .....A..C.....G.....A..G..T.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JX511966.1 .....A..C.....A.....A..G..C.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JX524447.1 .....A..C.....A.....A..G..T........AA.T..A..AG.GGT.A....T...TCA.....A...C.AT.G.....G.....
JX524443.1 .....A..C.....A.....A..G..T........AA.T..A..AG.GGT.A....T...TCA.....A...C.AT.G.....G.....
JX524444.1 .....A..C.....A.....A..G..T........AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JX524453.1 .....A..C.....A.....A..G..T.....C..AA.T..A..AG.GGA.A....T...TCA.....A...C.AT.G.....G.....
JQ347264.1 .....A..C.....A.....A..G..G.....C..AA.T..A..AG.GGA.A.....TCA.....A...C.AT.G.....G.....
KX247389.1 .....A.....C..A.....G.....GC..AT..G.T.ACA.....TCA.....TG.....C..CA.G..T.....A.
EU310300.1 .....T..A.G...CA.GG.A..AAAGG..T.CA....C.CA..GA.ATAC..A.AGG.T..TG.TG.AA....A..AA.C..CGC...CT...CT....
EU310294.1 .....T..A.G...CA.GG.A..AAAGG..T.CA....C.CA..GA.ATAC..A.AGG.T..TG.TG.AA....A..AA.C...GC...CT...CT....
EU310296.1 .....T..A.G...CA.GG.A..AAAGG..T.CA....C.CA..GA.ATAC..A.AGG.T..TG.TG.AA....A..AA.C..CGC...CT...CT....
EU310298.1 .....T..A.G...CA.GG.A..AAAGG..T.CA....C.CA..GA.ATAC..A.AGG.T..TG.TG.AA....A..AA.C...GC...CT...CT....

```
EU310292.1  .....T..A.G...CA.GG.A..AAAGG..T.CA....C.CA..GA.ATAC..A.AGG.T..TG.TG.AA....A...A.C..CGC...CT...CT....
EU310290.1  .....T..A.G...CA.GG.A..AAAGG..T.CA....C.CA..GA.ATAC..A.AGG.T..TG.TG.AA....A...A.T...GC...CT...CT....
EU310297.1  .....T..A.G...CA.GG.A..AAGAGG..T.CA....C.CAA.GA.ATAC..A.AGG.T..TG.TG.AA....A...A.C....C...CT.T.CT....
AY529714.1  .G.A..---.T...GA.AGGC.-AA.G...TGGTGT...GCCATCA.CTGCCC...TGAC..TG.T..AT.A..A------T..CAAA..T..C.TG.C.
KR026972.1
 Consensus  aaagt.gc.a.agt..t..c.gc..c.caa.t..a.ttta...a..a.....a.a...t.tt..a..aa.ctgt.aa..t.ctt..tga..gcaa.cc.g
            801                                          849
    Tomato
MT018563.1  TTCAAA---GAAGAG---CTCAAAGCCTAGTACATCTATAGAGGTGTAA
MT018562.1  TTCAAA---AAAGAG---CTCAAAGCCTAGTACATCTATAGAGGTGTAA
MT018561.1  TTCAAA---AAAGAG---CTCAAAGCCTAGTACATCTATAGAGGTGTAA
KU941832.1  TTCAAA---GAAGAG---CTCAAAGCCTAGTACTTCTAGATAA
EU216023.1  TTCAAA---GAAGAA---CTCAAAGCCTGGTCCTTCGCTGGAAGTGTAA
EU095940.1  TTCAAA---GAAGAG---CTCAAAGCCTAGCCCTTCTCTGGAATTGTAA
HG917416.1  TTC
JQ406584.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JQ406583.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524451.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524445.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524446.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524448.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX511966.1  TTCTAAGAAGAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524447.1  TTCTAAGAAAAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524443.1  TTCTAAGAAAAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524444.1  TTCTAAGAAAAAGAG---TTCAAAGGCTGGTCCTTCGCTGGAATTGTAA
JX524453.1  TTCTAAGAAGAAGAG---TTCAAAAGCTGGTCCTTCGCTGGAATTGTAA
JQ347264.1  TTCTAAGAAGAAGAG---TTCAAAGTTTGGTCCTTCGCTGGAATTGTAA
KX247389.1  CTCTAA---AAAGAG---CTCAAAGGCCAGCACTTCTTTGGAAGTGTAA
EU310300.1  TTCTAAACCCAAAAATCCCTCTAAGAAAGACAGATATAACTAG
EU310294.1  TTCTAAACCCAAAAATCCCTCCAAGAAAGACAGATATAACTAG
EU310296.1  TTCTAAACCTAAAAATCCCTCCAAGAAGGACAGATATAACTAG
EU310298.1  TTCTAAACCCAAAAATCCCTCTAAGAAGGACAGATACAACTAG
EU310292.1  TTCTAAACCCAAAAATCCCTCCAAGAAGGACAGATATAACTAG
EU310290.1  TTCTAAACCCAAAAATCCCTCTAAGAAGGACAGATACAACTAG
EU310297.1  TTCTAAACCCAAAAATCCCTCCAAGAAAGACAGATATAACTAG
AY529714.1
KR026972.1
 Consensus .tc.........................................
```

4.8 Variability analysis of CaCV

Various isolates of CaCV originated from India were taken for variability analysis. After analysis our CaCV isolate was found most closely related with CaCV isolate of Himanchal Pradesh HG917416.1 at nucleotide level using a tool named MEGA (Fig 8).

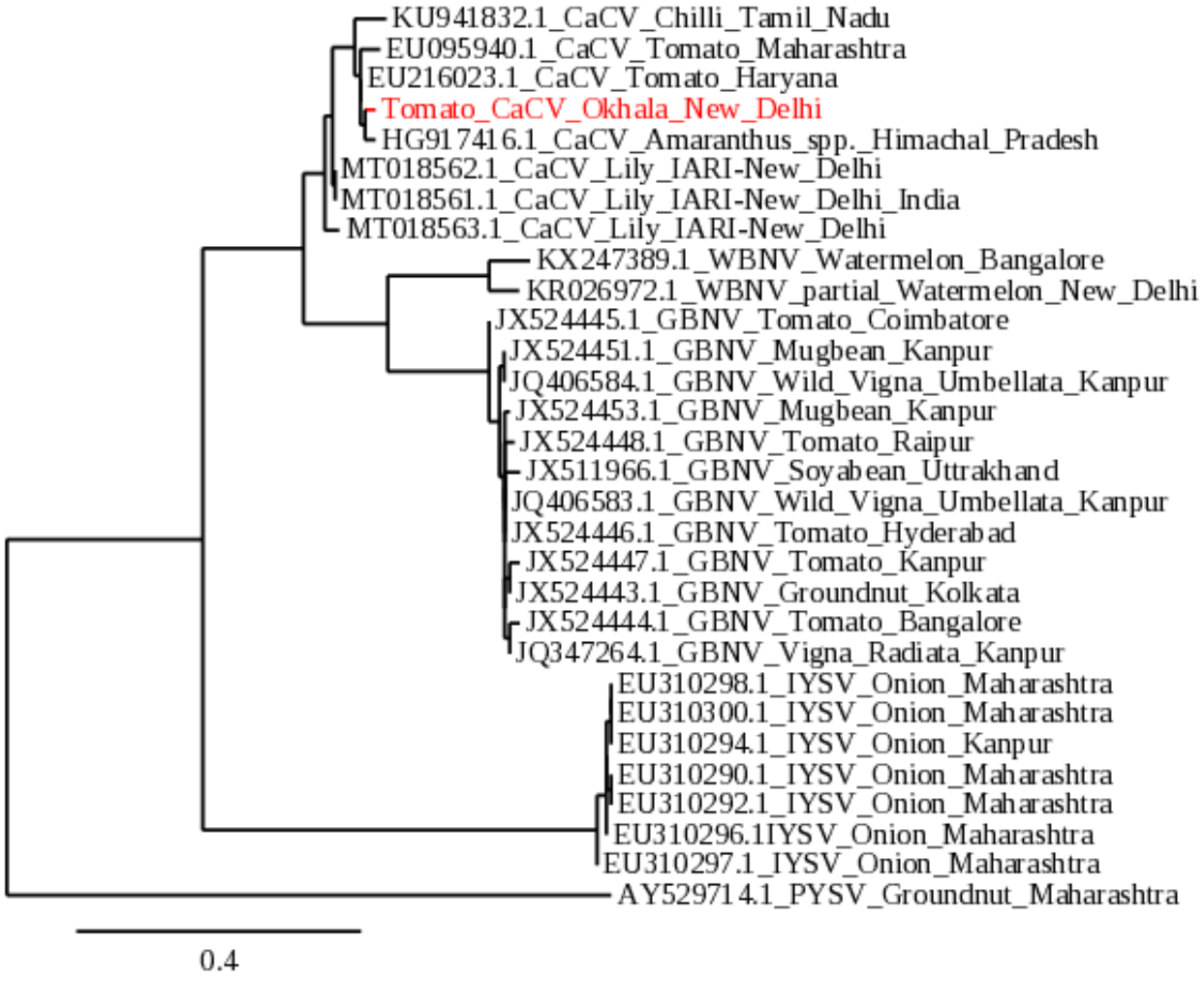


Figure 8: Phylogenetic tree based on deduced nucleotide sequences of Np gene of Tospovirus isolates reported from different parts of India.

5 CONCLUSION

The only plant-infecting viruses mostly in family bunyaviridae member of the genus tospovirus, and their economic impact on global vegetable output is significant (German et al., 1992). (Basavaraj et al., 2017; Jain et al., 2005; Ravi et al., 2006). Over the past 10 to 15 years, tospoviruses have become a significant hazard to vegetable crops in India. A new case of the Tospovirus genus' Capsicum chlorosis virus in tomato was discovered in northern India during the 2007 wet season. Compared to healthy plants, the infected ones grew more slowly (S. Kunkalikar, Poojari, Rajagopalan, et al., 2007).

Comparative biological and molecular characterisation of CaCV has been conducted. Tomato bud blight is a natural illness that causes signs on the fruits such as chlorotic ring patches or discoloured and malformed fruits.

CaCV was amplified by RT-PCR. Symptomatic peel samples showed an amplification of ~840bp which confirms the presence of Tospovirus associated with the disease. Amplified products were sequenced and the sequences were analysed.

The sequenced region contained a single open reading frame of 813 nucleotides that could potentially code for a coat protein of 246 amino acids.

Various isolates of CaCV originated from India were taken for variability analysis. After analysis our CaCV isolate was found most closely related with CaCV isolate of Himanchal Pradesh (HG917416.1) at nucleotide level.

Studies based on symptomatology, PCR and sequencing studies and comparison of NP gene sequence homology of tomato Tospovirus shared maximum sequence identity with other CaCV isolates at nucleotide (96-99%) and amino acid (95-99%) levels. Sequence homology with CaCV of other isolates in different places indicated that the present bud blight disease caused by CaCV on tomato could be designated as Calcium Chlorosis virus infecting tomato crop.

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