Study on Citrus Tristeza Virus from North East India and Development of Immunological Techniques of CTV Detection

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ABSTRACT

In terms of international trade value and world fruit production, citrus is the most valuable fruit crop. In India, citrus, after mango and banana, is the third major horticultural crop. In most citrus-growing regions, it is typically commercially grown as a monoculture. Therefore, any onset of this crop epidemic will lead to tremendous destruction of crops. Citrus trees, being a perennial and evergreen crop, are affected during their life cycle by a variety of biotic and abiotic factors. A viral disease affecting citrus,' tristeza' caused by Citrus tristeza virus (CTV) has the greatest effect worldwide among the biotic variables. More than 100 million citrus plants, with more than 400 million grafted citrus trees already at risk of CTV infection, have already been destroyed by this virus around the world. More than one million trees have been killed by this viral infection in India alone. There are multiple pathotypes of CTV, which display distinct symptoms along with symptomless strains on various citrus hosts. It is often associated with three manifestations: rapid deterioration, stem pitting, and yellow seedlings. The virus is confined to phloem and the disease continues to spread to new areas, either through the dissemination of infected buds or by semi-persistent transmission by various species of aphids. Owing to numerous and regular inoculations by aphids and the fallible existence of its RNA polymerase, infected citrus plants often harbour a large number of distinct CTV strains. As such, knowledge of the genetic diversity and geographical distribution of CTV plays a crucial role in the understanding of virus epidemiology, contributing to the proper diagnosis and development of long-term management strategies.

Key Words – *CTV, Techniques*

INTRODUCTION

Citrus trees and shrubs of the Rutaceae family are flowering, bearing a fruit known as the citrus fruit. Grapefruits, lemons, pomelos, oranges and other small fruits such as tangerines, mandarins, clementines and satsuma are included in the citrus fruits. The major group, however, is oranges, accounting for around 70 percent of the total production of citrus fruit. Citrus is grown in as

many as 140 countries as a horticultural crop, producing 124 million tonnes of fruit annually in 2016. (FAO, 2016). India is one of the largest contributors to citrus production, securing third place in the world ranking (FAO, 2016) with an annual production of approximately 13 million tonnes of citrus fruit produced in 2017 on approximately 1 million ha of land.

The Northeastern (NE) part of India holds an important role in the occurrence and diversity of citrus in the world In this area of India, 17 citrus species are cultivated and interestingly eight of these species originated from there themselves (Hore and Barua, 2004). In a range of 5-15 m in height, citrus plants are either small to moderate sized trees or large shrubs with spiny shoots and evergreen leaves. The flowers are mostly tiny or solitary corymbs, 2-4 cm in diameter. The fruits are hesperidia, a specialized berry with a leathery pericarp of approximately 4-20 cm in diameter. The pericarp contains flavonoids, limuloids and high levels of itric acid, which are the usual sharp flavours of citrus fruits (Nogata et al., 2006). With a minimum of 60 cm of well-drained topsoil with a slope of up to 15%, citrus trees require loamy to sandy loamy soil. Trees are susceptible to frost and can withstand high temperatures, but it varies depending on variety, tree age and health (Srivastava and Singh, 2009). Within two or three years of transplanting, the grafted trees bear fruit, but seed-grown trees take seven or more years to produce fruit. There are several prevalent biotic and abiotic factors that restrict the growth and yield of citrus plants. In most citrus growing regions, citrus is normally cultivated as a monoculture crop. Therefore, the onset of this crop epidemic could lead to enormous destruction of crops. Citrus canker, gummosis, powdery mildew, tristeza, dwarf, crinky leaf, etc. are the major citrus diseases that cause significant crop loss (Tennant et al., 2009). Among the biotic stresses, in several commercial citrus species, viruses and a fastidious greening pathogen cause major yield losses from the nursery to the fruit bearing point (Ahlawat et al., 1992).

OBJECTIVE

1. Survey and collection of CTV infected citrus samples from Northeastern states of India

2. Cloning and expression of the coat protein gene in Escherichia coli

Origin and distribution

Citrus has been associated with mankind from time immemorial. The generic name was originated from Latin, which referred to the present day plant “Citron” (C. medica) or “Thuja” a conifer tree It is also related to the ancient Greek word for cedar, κέδρος (kédros), may be because of the similar smell of cedar leave and fruit with that of citrus (Akhter et al., 2012). Using genetic mapping of chloroplasts, it has been estimated that approximately 7 million years ago, citrus plants diverged into two groups: the main genus, Citrus and the ancestors of the trifoliate orange (Poncirus) The latter group is still so closely related with the main group that it can be used for hybridizing with all other citrus. A recent whole genome based study concluded that citrus plants originated in the foothills of Himalayas, comprising state of Assam, India, western Yunnan, China, and northern Myanmar (Wu et al., 2018).

Citrus medica L. (citron) is considered to be the ancestor of citrus trees in Europe and Middle East which was introduced by Alexander the Great from India into Greece, Turkey, and North Africa in the late 4th century BC (D’Onghia et al., 2009). Citrus is a common term and indicates

the genus Citrus of the Rutaceae family. Today, citrus is distributed all over the world from south through the east Indian Archipelago to New Guinea, west Pakistan to north-central China as well as the Bismarck Archipelago, New Caledonia, northeastern Australia, Melanesia and the western Polynesian islands covering more than 140 countries (Wu et al., 2018). Most of the todays common citrus fruits, such as oranges, lemons, grapefruit, limes, and so on, were produced by crossing of the three original citrus species, which include mandarin orange, pummelo and citron (Velasco and Licciardello, 2014).

Citrus Tristeza Virus and its impact on citrus cultivation

The annual average growth rate in the citrus sector was around 20% annually from 1961 to 2012-13 which reduced to 4% in the last five years indicating that further increase in production shall only come from increase in per ha productivity as there is not much scope in increasing the area under cultivation (Central Citrus Research Institute, 2015). The increase in per ha depends upon the biotic and a biotic factors that influence the growth and health of a crop. Being a perennial and evergreen crop, citrus trees are subjected to a number of biotic and biotic factors throughout the annual cycle. Among the biotic factors, a viral disease affecting citrus, 'trustees' caused by Citrus tristeza virus (CTV) has the greatest impact worldwide (Moreno et al., 2008). Of all the known plant viruses, CTV has the largest and the most complex genome. It has a single stranded, non-segmented, positive-sense RNA genome of approximately 20 kb size and belongs to the genus, Closter virus. The CTV genome is covered by two capsid proteins of 25 kDa (CP) and 27 kDa (CPm) that coat 95% and 5% of the virion length, respectively. The genome has 12 open reading frames (ORFs), potentially encoding at least 19 different proteins. The complete sequence of several CTV isolates has been reported, including the sequence of a typical mild Spanish CTV isolate.

The virus is phloem limited and is transmitted readily to long distance via infected graft and within the fields semi-persistently by the main citrus visiting aphid species: Toxoptera citricida, Aphis gossypi, Aphis spiraecola and Toxoptera aurantii (Bar-Joseph and Lee, 1989). CTV infects nearly all citrus species, relatives and hybrids, and is widely distributed in all citrus growing regions of India viz. northwest, central northeast and south with an estimated disease incidence of upto 90% (Biswas, 2008). This virus is primarily associated with quick decline (QD) of citrus species grafted on sour orange rootstocks and stem pitting (SP) of grapefruit, pummelo and some sweet orange varieties irrespective of rootstock. A third disease, seedling yellow (SY) is also caused by CTV in young sour orange, lemon and grapefruit plants under experimental conditions. Damages caused by severe QD and SP isolates can be extremely devastating; however, trees with mild isolates show some productivity for a certain period (Bar-Joseph et al., 2002).

There are more than 100 million citrus trees that have already been killed by this virus across the globe with more than 400 million grafted citrus trees presently at the risk of CTV infection In India alone, more than one million trees were destroyed by the infection of this virus All the commercial cultivars of India are being reported to be infected by CTV The report mentioned that the incidence is highest (47.10-56%) in the east and NE India, which include Assam, Meghalaya, Sikkim and Darjeeling followed by the southern states of Andhra Pradesh and Karnataka (36-50%). However, in the northern states of Uttarakhand, Delhi and Punjab, the occurrence percentage ranges from 16-60%. In NE India, this virus was first reported in the year

1989 but till date only a few research on citrus decline due to CTV have been focused in this part of the country.

The CTV-infected young trees tend to flower a year or two earlier than the healthy plants with smaller fruits that develop color early. Moreover, the disease keeps spreading into new areas, either by propagation of infected buds or semi-persistently by different aphid species (Bar-Joseph and Lee, 1989). Unfortunately, T. citricida, which is the most efficient transmitter of CTV is prevalent in the NE India There is a need for CTV isolates to become adapted to local aphid vector populations which explains why in many countries there is a lag period (sometimes more than 30 years) between CTV introduction and noticeable field spread. Therefore, in this regard it is worth mentioning that failure to eradicate early CTV-infected trees results in epidemics. In the same context, during our survey of mandarin orchards in NE India, we came across many low income farmers who have relinquished citriculture and adapted other fruit crop as thousands of trees have either died or are in various stages of decline with premature falling of fruits rendering them unproductive and resulting in considerable losses. The unit cost of setting up a mandarin orchard estimates to Rs. 140700.00 /ha /5 years

(http://nhb.gov.in/report_files/orange/ORANGE.htm). Therefore, early diagnosis of the viral infection and effective management of CTV is imperative for promoting viticulture.

Novel pathogen city traits are continuously being inculcated by recombination of RNA viruses which have been extensively documented (Chare and Holmes, 2006). Therefore, knowledge on the genetic diversity of CTV and its geographical distribution play a vital role in understanding the epidemiology of the virus resulting in proper diagnosis and development of long term management strategies.

Diagnosis of CTV viral infection

CTV is one of the biggest oppressions to the global citrus industry and a prime contributor in decreasing citrus production. It has several types of path types which show different symptoms on different citrus hosts, along with symptomless strains. The symptoms of this disease often submerge with other bacterial diseases and those of nutrient deficiency and in most cases the symptoms appear only when the trees have matured. But, until then the growers make huge investment in maintenance of the diseased plants. Bio-indexing has been considered a primary diagnostic method for diagnosis of CTV infection and bio-characterization for the past seven decades However, it is time consuming and may take a month to a year or even longer to get complete results. Light microscopy and electron microscopy (EM) were also used for detection of CTV (Garnsey et al., 1980) but availability of the microscopes for routine use is not feasible every time.

In the recent years, CTV research has been concentrated on its detection and diagnosis through serological methods, molecular techniques, characterization by sequencing and phylogenetic analysis. Antisera to whole virus was used for detection of CTV infection by SDS-double immunodiffusion (Gonsalves et al., 1978), serologically specific electron microscopy (SSEM) (Garnsey et al., 1980), radioimmunoassay (Lee et al., 1981), dot immunobinding assay (Rocha-Peña et al., 1991) and enzyme linked immunosorbent assay (ELISA) (Bar-Joseph et al., 1979). A single amino acid change in the 124th position of CP gene was used to differentiate between the

severe and the mild strain. Phenylalanine is present in the severe strains whereas it is tyrosine for the mild strain (Huang et al., 2004; Pappu et al., 1993). Therefore, production of MCA-13 monoclonal antibody led to the distinction between severe and mild strains of CTV by ELISA (Permar et al., 1990). Until now, ELISA has been widely used to detect CTV infection (Borah et al., 2012). However, ELISA too has some drawbacks, including time-consuming and tedious washing and incubation steps. Polymerase chain reaction (PCR) based detection has been found to be more sensitive and reliable and can be performed within 6-8 h but requires expensive reagents and machine. As a fact, the detection methods described above do not meet on-site testing and are also not suitable for detection of large population of samples due to high operating cost.

Therefore, the need of the hour is to develop a rapid, cost effective and simple detection method with high specificity and sensitivity to detect CTV infection in the field at the seedling stage right after grafting so that no further investment is made for maintenance of the diseased plant and also for production of clean planting material. Viral antigen purification is difficult as CTV is restricted to phloem tissue (Lee et al., 1987), therefore bacterially expressed coat protein is an alternative The molecular approaches for tristeza research in India mainly revolves around ELISA, RT-PCR and cloning of the gene but no work on expression of the viral protein in bacterial system and its purification has yet been reported. However, research has been conducted on prevalence and diversity study of CTV isolates from different parts of the country The aim of the present research is to express the viral coat protein and produce polyclonal antibodies emphasizing the fact that an immunodiagnostic kit can be developed for rapid detection of CTV in the field and also for virus indexing of plant material.

Citrus fruits

Citrus fruits, belonging to the genus Citrus of Rutaceae family, are recognized for their refreshing fragrance and thirst quenching ability. The fruits contain adequate minerals, dietary fiber and vitamin C in accordance with recommended dietary allowance (Ladaniya, 2008). In addition to this, citrus fruits also contain neutraceutically important photochemical like flavanones, arytenoids and limonoids. Growth of the citrus industry, including rapid development of the processing technology of frozen concentrated orange juice after World War II, has greatly expanded with international trade and steadily increased the consumption of citrus fruits and their products during the past several decades (Liu et al., 2012). Citrus industry generates employment to millions of people around the globe in different maneuvers like harvesting, handling, transportation, storage and marketing operations. Apart from consuming raw citrus fruits and in the form of fresh or packaged juice, efforts have also been made for developing alcoholic beverages by fermentation. Many investigations have been undertaken to explore the potentials of mandarin oranges to produce wine (Gupta et al., 2009; Koh et al., 1989). The origin of citrus is generally believed to be southeast Asia (Wu et al., 2018). The NE India, being an important part of Indo-Burma biodiversity hotspot, is considered as one of the origins of various citrus species (Singh et al., 2016). Citrus medica L. (citron), regarded as the ancestor of the citrus trees in Middle East and Europe is believed to have been introduced by Alexander the Great from India into Greece, Turkey, and North Africa in the late 4th century BC Other specific regions for origin of citrus include the Yunnan province of southwest China and Myanmar (Wu et al., 2018). However, there are several other interesting legends on the history of origin of citrus. Some historians contemplate Australia, New Caledonia (off eastern

Australia), and New Guinea as the true origins of citrus. Citrus is believed to be initially introduced by Spanish and Portuguese explorers in America leading to the appearance of orchards in Florida and California in 1655 and 1769, respectively. Since then, the commercial production, processing, and global trade of citrus have significantly improved, making citrus the most important fruit crop globally (Liu et al., 2012).

Strain characterization and genetic variation

The elucidation of earliest CTV genomes: T36 from Florida VT from Israel T385 from Spain and T30 from Florida indicated that these isolates diverged from each other with two distinct trajectories: one was the VT-like and T30-like isolates, and the other T36-like Over the past decade, the additional sequencing of novel isolates revealed far higher global CTV diversity than the previously imagined, and that new genotypes have either evolved by recombination or diverged from the ancestral population. The difficulty in the identification of new CTV genotypes lies in the asymmetry in the genetic variation which is unevenly distributed along the CTV gRNA, the 3′ UTR being the most conserved region with more than 95% identity between the isolates, and the 5′ UTR being the most variable, with identity values as low as 44-45% between sequence types I and III (López et al., 1998).

For instance, the T3 and NZ-B18 isolates could be distinguished from each other and also from VT only on the basis of their 5′ end of the genome which are all otherwise homologous when their 3′ sgRNA coding genes are compared (Harper, 2013). Classification of CTV genotypes is further complicated by the existence of recombinant isolates such as SY568 a stem-pitting isolate from California (Vives et al., 2005) and Hawaiian isolate HA16-5 (Melzer et al., 2010). Advancement in sequencing methodologies revealed that different isolates of CTV fall into a series of groups based on their sequence similarity, which were termed as strains. The analysis of 38 full-length sequences of 32 different CTV isolates demonstrated that the sequences could be characterized into seven defined sequence groups that differed by >7.5% at the nucleotide level on comparing the whole genomes (Harper, 2013). The strains are designated as T30, a mild isolate from Florida (Albiach-Martí et al., 2000b); T36, a decline isolate from Florida (Karasev et al., 1995); VT, a decline isolate from Israel (Mawassi et al., 1996); RB, a resisitance breaking isolate from New Zealand (Harper et al., 2010); T3 (GenBank Accession no. KC525952) from Florida, T-68 (Harper, 2013) and HA16-5, a recombinant Hawaiian isolate (Melzer et al., 2010). Overall, sequence comparisons between CTV isolates of different geographical origin and pathogenicity characteristics show a high degree of conservation between CTV genomes separated in time and space, with a limited repertoire of genotypes (Albiach-Martí et al., 2000b; Ruiz-Ruiz et al., 2006; Vives et al., 1999) and a population structure variable between isolates, with some consisting of a predominant sequence and some closely related variants, and others having a complex structure with highly divergent sequence variants (Ayllón et al., 2006; Vives et al., 2005).

Novel pathogen city traits are continuously being inculcated by recombination of RNA viruses which have been extensively documented (Chare and Holmes, 2006). Factors shaping CTV populations in the field include mutation, recombination events between diverged sequence variants, selection, genetic drift and gene flow due to repeated inoculation of field trees and movement of infected buds between regions (Moreno et al., 2008). The repair mechanism to correct transcription errors during CTV replication is erroneous due the fallible nature of its

RNA polymerase which leads to accumulation of any change or mutation occurring during replication which may remain undetected resulting in certain changes in biological or biochemical properties. The replicating CTV in symptomless or tolerant hosts may eventually give rise to new severe strains or to highly transmissible severe variants when propagated as new varieties Mutation due the error-prone nature of RdRp is the basic mechanism generating diversity (Domingo, 1994). Therefore, knowledge on the genetic diversity of CTV and its geographical distribution plays a vital role in understanding the epidemiology of the virus resulting in proper diagnosis and development of long term management strategies. Incongruent phylogenetic relationships between CTV isolates in different gRNA regions indicated that homologous recombination between diverged sequence variants may be a frequent phenomenon in CTV isolates Recombination could repair in some cases lethal or biologically disadvantageous mutations, providing opportunities for rapid evolution (Moreno et al., 2008). In the same context, a number studies have been conducted in the India to identify the genetic diversity and recombination analysis of CTV strains prevalent in different geographical zones based on the phylogenetic study of the CTV-CP gene and that of the 5´ORF 1a region using MEGA recombination detection program (RDP) Other methods for phylogenetic analysis include PHYLIP programs DNAML and NEIGHBOR (Rubio et al., 2001) and that of recombination analysis include Genetic Algorithms for Recombination Detection (GARD), SimPlot software GENECONV and PHYLPRO programs (Rubio et al., 2001). Hotspot for homologous recombination were reported to have an AU-rich stretch of 8-89 nucleotides which is shared by both parental sequences and flanked by GC and AU rich regions upstream and downstream, respectively (Hilf et al., 1995). The complete genome sequence of a decline CTV isolate, Kpg3, of the Darjeeling hills of the Northeastern Himalayan region of India was reported (Biswas et al., 2012b). Phylogenetic analysis revealed that the Kpg3 genome is closely related to isolate VT and distantly to T36 and B165. Also, recombination analysis indicated that Kpg3 is recombinant and originated through multiple recombination events in which parts of the genome were exchanged between divergent CTV sequences Recently, the complete genome sequence of a resistance breaking CTV isolate DSST-17 was reported from Uruguay which presents two recombination events involving T36 and B165-like fragments.

CONCLUSION

In the present study, an attempt was made to collect, screen and characterize CTV from three different states of NE India. This was followed by development of two immunological methods for detection of CTV infection in plants. The developed dipstick can be used for routine diagnosis of CTV infection in the field samples right at the seedling stage. This will help farmers and quarantine agencies to take up CTV management decisions at an early stage to check the spread of disease and avoid financial losses which might have occurred due to maintenance of the diseased plants, since in most cases the symptoms appear when the trees mature. The salient findings of the present study are summarized here under.

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