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CITRUS TRISTEZA VIRUS: ITS RESEARCH STATUS AND FUTURE PROSPECTS IN PAKISTAN

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A B S T R A C T

Citrus tristeza virus (*CTV*) produces the most damaging disease of citrus worldwide and is a continuous threat to the citrus industry. It is primarily spread through infected root-stock and brown citrus aphid in a semi-persistent manner. Infected trees produce small sized and poor quality fruit, and in severe cases tree dies. Sour orange rootstock is highly susceptible to *CTV*. Several mild and severe strains have been identified which produce from invisible- to- visible symptoms in diseased plants. Existence of multiple *CTV* strains in nature is a challenge for *CTV* management. Symptomless infection in certain citrus species results in the inoculum build up and further spread of the disease. Breeding for *CTV* resistance genes is a long and time-consuming process that takes 8-10 years before a *CTV* resistant variety is developed. Genomic manipulation of *CTV* genome is challenging due to its larger genome size. Modern biotechnological tools can be used to control this disease and to prevent its spread in the future. However, *CTV* offers a valuable research tool for its role as a stable marker in genetic transformation of plants. This review highlights the challenges in developing resistant citrus cultivars against *CTV* and future prospects of *CTV* and is an update to the research status of *CTV*.

Keywords: *Citrus tristeza virus*, resistance breeding, disease management, genetic transformation.

INTRODUCTION

Citrus (*Citrus* L.) is an important fruit tree grown worldwide. Citrus is a rich source of phytochemicals including vitamin C, potassium, folic acid, pectin and riboflavin (Ahmed and Azmat, 2019). Citrus fruit extracts and flavonoids show a range of beneficial health properties due to their high phenolic and antioxidant content (Singh *et al*., 2020). In Pakistan, citrus is grown on a large area and Pakistan is the 13th largest citrus producer (FAO, 2020). During 2018-19, area under citrus species in Pakistan was 1,817,000 hectares. Punjab is major citrus-producing province in which 170,000 hectares of land is under citrus followed by Sindh (4000 hectares), KPK (4000 hectares) and Balochistan (1700 hectares) (FAOSTAT, 2018). Citrus produced in Pakistan is mainly consumed locally or exported to fresh fruit markets in nearby countries including Russia, UAE etc. (Ahmad *et al*., 2021).

The *citrus tristeza virus* is unique among hundreds of other viruses. The word tristeza meaning "sadness"" alternatively the meaning of this disease is "rotting of rootlets". *Citrus tristeza virus* is a deadline syndrome, mainly spread through infected root stock and several species of aphid also transmit this disease but its transmission to new plant variety via seed has not been observed. Aphid spp. known as *Toxoptera citricida* (brown citrus aphid) is most efficient vector in transmitting *CTV*. Inhabitant's places of this virus are mainly observed in phloem cells of some species of Rutaceae. Moreover, spread of this disease to other countries mainly occurs during transport of complete citrus plant and soil to other lands at the end of nineteenth century. Due to increased commercial and botanical interest in citrus, movement of plants from Asia to other countries increased, which lead to the main spread of this virus worldwide. Due

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to this exchange citrus interact with new host varieties and combinations on different climatic and environment conditions. Depending on *Citrus tristeza virus* new strains, it mainly causes three different types of syndromes, in which two are most important 1: Quick decline, 2: Stem pitting, 3: seedling yellows. *CTV* induce obliteration necrosis and collapse of the sieve tube and companion cells closed to the bud union, which mainly leads to the production of non-essential phloem. The useless phloem cause restriction and blockage in the transport of water supply to plant and plant starts to wilt due to deficient supply of water and minerals. The virology studies related to *CTV* mainly started after research conducted in brazil by a famous scientist Kitajima who had observed tiny particles in association with infected plant roots under electron microscope. Trees infected with this virus mainly exhibit symptoms like foliage in light green color, stunted growth and some leaf drop. Girdling that occur near the bud union inhibit the transport of starch to the rootlets. Feeder roots starts drying from tips toward main roots.

Citrus tristeza virus (*CTV*) has become a serious threat to citrus trees globally. Since 1920, more than 100 million citrus trees have been destroyed by quick decline due to *CTV* in North and South America. The virus is a member of the Closteroviridae family and genus Closterovirus. The virus is spread by several aphid species in semi-persistent manner in nature, however, brown citrus aphid, *Toxoptera citricida* has been considered as an efficient vector. *CTV* acquisition time from infected hosts ranges from few seconds to 30 min, followed by incubation period of several hours inside the insect until the aphid can transmit it to a new host. Viruliferous aphids can retain the virus particles for 2 days and infect as many plants they feed on during this period. Host susceptibility, virus strain and environmental factors influence the transmission efficiency of *CTV* by aphids.

The genome of *CTV* is single stranded with positive sense RNA of 19.3 kb in size. *CTV* have 12 open reading frames (ORFs) encoding 19 different proteins. Virus particles are flexuous, filamentous, 2,000 x 11 nm in size and have two coat proteins (major coat protein and minor coat protein) (Karasev, 2000). Protein p33 plays a role in extending host range of *CTV*. The protein localizes at plasmodesmata and forms extracellular tubules. Protein p23, p25 and p20 acts as RNA silencing suppressors in the host. Other proteins p13, p18 and p33 are involved in producing systemic infection and stem-pitting symptoms in some hosts. Long-distance movement through sieve tubes is predominant during systemic infection in comparison to cell-to-cell movement; however, in other plant viruses' intercellular movement is predominant. Virions travel through sieve tubes and rarely enter in adjacent parenchyma cells where virus replication occurs. In few hosts, cell-to-cell movement occurs in only a small cluster of nearby cells, while in others there is no cell-to-cell movement. P13, p18 and p33 are not essential for infection of most hosts but play role in infection of some citrus species. In sour orange, gene p23 is responsible for induction of seedling yellows (SY) symptoms.

CTV is widespread in citrus-growing areas of Pakistan and resulting significant yield losses each year. Three genes of the virus genome (p33, p18, and p13) are not essential for infection but they are known to play different roles during the infection process. Possibly *CTV* acquired these genes during the evolution process. *CTV* is widespread in citrus orchards in Pakistan. Infected tree samples collected from Punjab showed maximum disease incidence in sweet orange followed by mandarin and grapefruit. Mixed infection of *CTV* isolates was found very common in the field trees in Pakistan. Since the first report of *CTV* in 1988, it has been reported from different orchards in Pakistan (Naqvi *et al*., 2015; Naseem *et al*., 2016; Atta *et al*., 2019). *CTV* has proved as research tool for genetic transformation and expression of sequences of interest in the target genome. It is useful vector for the stable transformation of perennial trees with foreign genes over many years. Several mild isolates of *CTV* which don't cause disease in citrus can be used as valuable vectors for genetic transformation of citrus and other perennial trees. The *CTV* vector can be used to express known sequences or mute host genes in order to protect or alter already-existing trees, or it can be used to evaluate candidate genes for potential advantageous activities in citrus. *CTV*-derived vectors are being tested for their use in genetic engineering of citrus for managing citrus greening disease.

***CTV* plant Symptoms:** *CTV* produces a broad range of symptoms in citrus plants depending on the type of *CTV*, host genotype and rootstock scion combination. Environmental conditions also influence the development of symptoms. However, three main symptoms are observed because of *CTV* infection. (Figure 1).

a) Quick Decline: The quick decline occurs when the tree is affected by *CTV*-SY strain or *CTV*-T strain. As a result, the tree starts wilting and ultimately dies within few months (Figure 1). Sweet orange, grapefruit and mandarin develop quick

decline when grafted on sour orange rootstock, but use of resistant rootstock results in reversal of quick decline.

b) Seedling Yellows: This is another important symptom that develops in self-rooted citrus plants. As a result, yellowing, stunting of trees, dieback of branches occurs and growth of trees is halted. Seedling yellows is sometimes transient; if trees are grafted on resistant rootstock, the trees recover immediately (Fraser *et al*., 1953; Albiach-Marti *et al*., 2010) (Figure 1). However, the mechanism of induction of seedling yellows symptoms is not understood yet. The p23 gene has been found to have a role in the induction of seedling yellows in sour orange.

c) Stem pitting: Stem pitting occurs in most citrus varieties even if grafted on resistant rootstock however few varieties are resistant to stem pitting. In stem pitting, vascular differentiation is affected and plants develop pits on their trunks. The trees bear profuse flowering, small and misshaped fruits. (Figure 1).

Genetic diversity of *CTV* in major citrus growing countries: Great genetic diversity exists among *CTV* isolates in natural populations. Populations of *CTV* in nature indicate the existence of multiple strains which differ from each other in their aggressiveness on citrus hosts. Coat-protein coding region has been found best for studying genetic diversity among *CTV* isolates. Coat protein sequences were collected through FASTA and assembled to perform phylogenetic analysis for studying genetic diversity in *CTV* worldwide. *CTV* has many strains which differ from each other in their severity, and extent of damage, (Roberts *et al*., 2001).

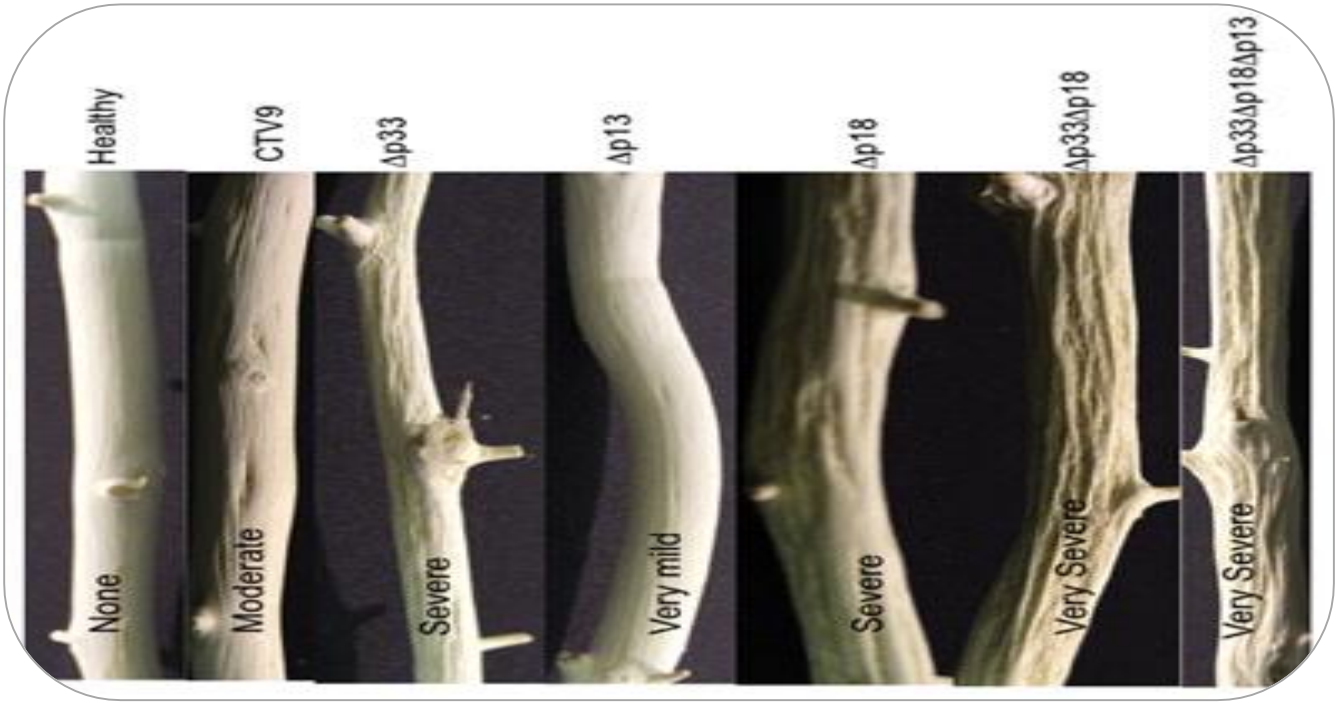

Figure 1. Stem pitting due to *Citrus tristeza virus* infection showing different severity on citrus seedlings (Photo taken from Dawson *et al*., 2013). Different varieties are showing difference in severity of stem pitting due to *CTV* on the infected plants.

Strategies for management of *Citrus tristeza* disease:

1. *CTV* tolerant rootstocks: An integrated disease management approach should be considered the most important management strategy for managing *CTV* in citrus orchards. *CTV* tolerant rootstocks (Any examples with reference) are being successfully used for managing *CTV*. Disease certification programs for testing *CTV*-free bud wood source trees can be established for maintaining disease free citrus seedlings (Garnsey *et al*., 2005). Micro-grafting of shoot tips is a well-known method that is used for virus and viroid elimination from citrus germplasm under greenhouse and glasshouse conditions. This technique has been successfully applied for the elimination of *CTV* in mandarins and sweet oranges (Krueger *et al*. 2003; Naz *et al*.2007).

2. Resistance development in citrus against *CTV*: Resistance genes of *CTV* are naturally occurring in wild citrus, *Poncirus trifoliata,* and offer a broad spectrum resistance towards all *CTV* isolates (Rubio *et al*., 2023. The gene covers 121 kb segment and comprises of ten candidate's resistance genes (Rubio *et al*., 2023). The R genes were cloned into vulnerable grapefruit types using

a binary vector based on Agrobacterium (Rai, 2006). Genes *R-2* and *R-3* were expressed in transformed plants and in *Poncirus trifoliata*, however in non-transformed controls five other R-genes were expressed. Northern blotting was carried out in virus-inoculated plants for testing infection (Rai, 2006). Each growth period comprised of 6-8 weeks and infection was absent or little virus spread was observed in R-2 lines. Initially unavailability of infection and its subsequent infection in *R-1* and *R-4* lines was observed (Rai, 2006). Resistance conferred by single dominant gene *CTV* is modified by a second gene (Ctm). Plants heterozygous for *CTV* showed resistance to most *CTV* isolates but in the absence of Ctm local virus movement was observed (Gmitter *et al*., 1996; Mestre *et al*., 1997). Resistance offered by *CTV* is constitutional, preventing some early stages of virus's infection process (Albiach-Marti *et al*., 2004). Development of new resistant varieties is slow process especially in case of perennial trees and due to limited availability genes of resistance. A fast-breeding technique was adopted for transferring *CTV* resistance genes from trifoliate orange into commercial citrus cultivars through overexpression of *CiFT* and MAS. By this technique one generation was achieved in one year. Analysis of linkage showed that *CTV* resistance was independently segregated in the BC progenies (Endo *et al*., 2020).

3. Genome engineering for *CTV* resistance: Vulnerability of citrus crop to various disease threats is a significant challenge for sustainable agriculture and demands genome improvement approaches to protect it from various pathogens (Peng *et al*., 2017). Different integrated disease control strategies including production of inoculum-free nursery stock, elimination programs and application of bactericides have previously been practiced (Graham *et al*., 2004). However, the high cost, human and animal health hazards and adverse environmental effects do not encourage their widespread applications.

Plant genetics breeding, mainly relies on introducing resistance genes to improve plant resistance. However, the high degree of genome heterozygosity is a shortcoming for the identification of active resistance genes and consequently hinders the molecular breeding programmes in citrus (Xu *et al*., 2013)

Recently, methods for genome editing and engineering have become more popular as a potential means of introducing desired genes into plants. Out of these genetic engineering technologies, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) CRISPR-associated nine (CRISPR/Cas9) systems have received particular attention due to its high simplicity, efficiency, and reproducibility. CRISPR/Cas has revolutionized modern plant breeding to produce virus-resistant cultivars (Ma *et al*., 2016). Several recent studies mostly have used CRISPR/Cas9 to incorporate resistance-gene in plants, either through cleaving of the viral genome and direct targeting, or by manipulating the host plant genome to enhance defense system of plants against viral pathogens (Zaidi *et al*., 2016). CRISPR/Cas9's advantage lies in its ability to eliminate the transgene from the enhanced variety, addressing public concerns surrounding the use of transgenic crops in food.

CRISPR-Cas technology has been used against *begomoviruses* and *closteroviruses* to produce virus-resistant plants. Targeted genetic engineering for precise genetic modifications propose a powerful tool for plant breeding and CRISPR/Cas9 system has been extensively used for genome editing in citrus (Jia and Wang, 2014; Belhaj *et al*., 2015; Jia *et al*., 2016; Peng *et al*., 2017). The system is widely adoptable and equally effective against both DNA and RNA viruses. Several studies indicate that CRISPR/Cas9 confers immunity in plants against DNA and RNA viruses either by disrupting viral genome or altering host plant genome (Ji *et al*., 2015; Zhang *et al*., 2018; Zhang *et al*., 2019). The CRISPR-Cas system was employed in a study to selectively target and dismantles the genome of the *Bean yellow dwarf virus* (BeYDV). In transient assays utilizing BeYDV-based replicons, it was observed that CRISPR-Cas sequences triggered mutations in the virus genome, leading to a decrease in the virus copy number. Plants modified with transgenes expressing CRISPR-Cas reagents, when exposed to BeYDV, exhibited diminished virus load and fewer symptoms, unveiling an innovative approach for developing resistance against *clostero viruses* (Baltes *et al*., 2015). Certain adaptations of CRISPR-Cas9 have been harnessed either to directly target the genome of plant RNA viruses or to target host genes/factors that facilitate viral proliferation (Hameed *et al*., 2019).

Citrus tristeza virus (*CTV*) is considered as a serious threat to be the cause of destructive impact on citrus production worldwide (Tabassum *et al*., 2013). Recent identification of FnCas9 from *Francisella novicida* (Zhang *et al*., 2018) and calibration of Cas variants namely Cas13a and Cas13d (Aman *et al*., 2018; Mahas *et al*., 2019) have emerged as new tools to target RNA virus genome and

create resistance against viruses. By taking advantage of this natural antiviral defense mechanism, genome editing technologies could be used to engineer the plant genome to trigger host-induced gene silencing (HIGS) by producing small interference RNA (siRNA) to target respective viral genome (Duan *et al*., 2012; Khalid *et al*., 2017).

Mostly plant viruses have RNA genomes and produce double stranded RNAs during their genome replication, which induce RNAi-mediated gene silencing in plants (Lindbo and Dougherty, 2005). By taking advantage of this natural antiviral defense mechanism, genome editing technologies could be used to engineer the plant genome to trigger host-induced gene silencing (HIGS) by producing small interference RNA (siRNA) to target the respective viral genome (Duan *et al*., 2012; Khalid *et al*., 2017). Recent identification of FnCas9 from Francisella novicida (Zhang *et al*., 2018) and calibration of Cas variants namely Cas13a and Cas13d (Aman *et al*., 2018; Mahas *et al*., 2019) have emerged as new tools to target RNA virus genome and create resistance against viruses.

Despite the robust advantages and usefulness of CRISPR/Cas system in genome editing, it has however been time consuming, involves complicated tissue culture and faces plant regeneration issues. Moreover, the prolonged retention and expression of CRISPR transgene cassettes in the plants may cause off-target effects and raises ethical concerns around the world. Very recently a genome editing technology has brought a breakthrough in plant genetic breeding by exploiting the shoot-root crosstalk through grafting (Geng *et al*., 2022). In a further refinement of the technology, a cassette consisting of Cas9 protein and a gRNA linked with mobile tRNA-like sequence (TLS) motifs is capable of moving from rootstock to shoots to generate stable transgene-free genome-edited plants in one generation (Yang *et al*., 2023). This genome editing system does not require CRISPR backbone. Citrus are highly amenable through grafting hence the technology could readily be applied for the genome engineering to fend off invading phages like *CTV*. Moreover, using the rootstock-scion grafting will offer resistance against soil borne diseases along with genome editing in shoots by transmissible Cas9/gRNA system. Additionally, besides CRISPR-Cas, techniques such as Zinc Finger Nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs) could be applied for genome editing with the goal of enhancing resistance against *CTV* in citrus, utilizing the web-based tool CHOPCHOP (Montague *et al*., 2014; Iqbal *et al*., 2016). The advances in biotechnological tools and the complete genome sequence of several citrus species will undoubtedly improve the breeding for citrus disease resistance with a much greater degree of precision (Sun *et al*., 2019).

4. RNA interference technology for *CTV* resistance: RNAi technology has proven effective in addressing Tomato yellow leaf curl virus (TYLCV)-beta satellite complexes, wherein hairpin RNAi constructs express double-stranded RNAs. Genes with homologues to IR, CP, and V2 replication-associated genes of TYLCV have been targeted through RNAi to silence the virus infection (Ammara *et al*. 2015). RNAi technology has also been used in citrus against *Diaphorina citri* which is a vector of citrus greening disease. The reduced capability of *D. citri* to fly would affordably retard the successful vectoring of citrus greening bacteria in-between the citrus plants planted in a grove (Hajeri *et al*., 2014). Citrus species resort to RNAi to lower *CTV* replication in infected cells and reduce systemic spread. There are three genes in *CTV* that act as silencing suppressors, including p20, p23, and CP (Lu *et al*., 2004). CP and p20 gene products retard cell-to-cell silencing and prevent spread of silencing signals and initiation of host defenses. Gene products of p23 and p20 avoid v and intracellular silencing. Genetically modified expression of p23 gene was found to increase the size and number of infection foci and thus *CTV* titer in sour oranges, and to spread virions from sweet orange plants and strict phloem-limitation in sour (Fagoaga *et al*., 2006). The p23 and CP genes play supplementary roles in the viral replication cycle, specifically in regulating the accumulation of negative strands and the encapsulation process, respectively. From an evolutionary standpoint, this competition has been compared to an ongoing "arms race." (Obbard *et al*., 2009).

5. Management *CTV* vector, *Toxoptera citricida*: *CTV* is transmitted semi- persistently by a brown citrus aphid, *T. citricida* (Roistacher and Bar-Joseph, 1987). The *T. citricida* have been proved to be widely distributed in Asia, Africa, North and South America, Europe, Australia, and Oceania. The population of *T. citricida* increases at a faster rate compared to the other aphid species. Due to the short life cycle, increased population, flying ability, and favorable environment are the key factors that may cause epidemics of *T. citricida.* Integrated management of *T. citricida* is crucial to managing *CTV* transmission by using biological agents, and bio pesticides, to minimize its

population in citrus orchards and nurseries.

Future Prospectus: Certified disease-free citrus nurseries should be established to control *CTV* spread. Disease-free root-stocks offer the best strategy for reducing disease epidemics in the future. Quarantine measures should be seriously taken to prevent the trade of symptomless hosts which carry the virus inoculum. The virus has an extensive host range, and all host plants should be inspected at the quarantine department during the trade of plant materials or products. Multiple isolates/strains co-infecting a single tree is a common phenomenon in citrus and it can result in the emergence of recombinant strains which are more virulent. Spreading through asymptomatic hosts in plant trade is another challenge. There is need is to find polygenic and durable resistance with a broad spectrum. Genetic techniques have been unsuccessful due to the poor transformation efficiency of citrus. Resistant gene from *P. trifoliata* should be interjected into commercial citrus cultivars in future breeding efforts. CRISP-R can be used to create resistance in citrus cultivars that cam provide long term protection against the disease. The new frontiers in CRISPR technology has opened up new ways for controlling *CTV*. Therefore, these technologies should be used to establish a disease-free plant for future cultivations.

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