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ARTICLE *in* JOURNAL OF INTEGRATIVE AGRICULTURE · MARCH 2012

Impact Factor: 0.83 · DOI: 10.1016/S2095-3119(12)60019-7

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March 2012

REVIEW

Distribution and Research Advances of *Citrus tristeza virus*

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Abstract

Citrus tristeza virus (CTV) is one of the most important causal agents of citrus diseases and exists as numerous strains. CTV is replicated in phloem cells of plants within the family Rutaceae and is transmitted by a few of aphid species. CTV epidemics have caused death of millions of citrus trees in many regions all over the world, where the sour orange (*Citrus aurantium*) was used as rootstock. Also the production of grapefruit (*C. paradisi*) and sweet orange (*C. sinensis*) has been affected by CTV strains. CTV gives uplift to three prominent syndromes, namely quick-decline (tristeza), stem-pitting and seedling-yellows. The disease is graft-transmissible in nature but not seed-transmitted. However, the tristeza disease in most citrus groves was a man-made problem created by the desire of horticulturists to introduce cultivars from other citrus growing areas. The utmost importance of the disease called for review articles in numbers of plant protection, epidemiology books, citriculture and proceedings. This review collects the information with respects to disease history, distribution host range, virus isolates association, identification and detection, transmission and management; especially on the current status of CTV prevailing and controlling in Pakistan. It provides valuable information for CTV disease and its controlling approaches.

Key words: *Citrus tristeza virus*, epidemic, status in Pakistan, control

INTRODUCTION AND DISEASE HISTORY

The center of origin of most citrus cultivars is perhaps unknown but the ancient relatives of citrus are native to China, the Southeast Asia, the Malay Archipelago, New Caledonia, and Australia, and then co-evolved with the host. CTV dispersal to new regions mainly occurs through the movement and propagation of the infected plants or infected buds and then locally it is spread by a few of aphid species (Bar-Joseph *et al.* 1989; Timmer *et al.* 2000). However there is no proper evidence of seed transmission. As seed transmission does not occur we have to suppose an early adaptation of CTV by aphid transmission. At the start, fruits or seeds of citrus were brought from the site of origin to other regions of the world (Zaragoza 2007), which gives evidences that CTV was not dispersed at that time. At the end of the 19th century, with an increased botanical and commercial interest and value in citrus by the horticulturists, citrus plants were introduced from Asia to other regions and vast exotic citrus species were exchanged between collections (Roistacher 1981). In 1836, a foot rot epidemic caused by oomycetes of the genus *Phytophthora* sp. started in the Azores and later affected the Mediterranean countries, destroyed sweet orange trees [*C. sinensis* (L.) Osb.], and led to the adoption of *Phytophthora* tolerant rootstocks. That was to propagate citrus varieties on sour orange (*C. aurantium* L.), a foot-rot-resistant rootstock, highly adaptable to

Received 18 March, 2011 Accepted 1 June, 2011
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all types of soil and producing good bearing and fruits of excellent quality. Sour orange soon became almost the tremendous rootstock in the Mediterranean area and then in America.

However, ultimately, the decision of using sour orange rootstock led to the dramatic effect that CTV has had on world citrus production. The new disease epidemic was dramatic for the citrus industry and caused the loss of almost 100 million trees propagated on sour orange and finally created the need for tristeza-tolerant rootstocks to rebuild a new citrus industry in the countries being affected. The most destructive epidemics of tristeza occurred in Argentina in 1930, then appeared in Brazil in 1937, was named tristeza. The epidemics occurred respectively in Ghana in 1938, California in 1939, Florida in 1951, Spain in 1957, Israel in 1970, and Venezuela in 1980, and the outbreaks have also been reported from other citrus groves, such as in Cyprus in 1989, Cuba in 1992, Mexico in 1995, the Dominican Republic in 1996, and Italy in 2002 (Bar-Joseph *et al.* 1989; Garnsey *et al*. 2000; Timmer *et al*. 2000; Gottwald *et al.* 2002; Davino *et al.* 2003). There are also indirect damages caused by CTV epidemics, such as the loss of sour orange rootstock which has the agronomic and horticultural qualities unmatched by any other rootstock, and the appearance of new problems related to the use of tristeza-tolerant rootstocks, such as the graft-transmissible diseases associated with the use of these rootstocks (Román *et al.* 2004).

Today CTV is widespread in Israel, Morocco, India, China, Japan, Pakistan, Iran, Syria, Egypt, southern California and Florida of USA, Argentina, Brazil, South Africa, Tanzania, Australia, and southern Spain, and is moving into northern Spain (EPPO 2006) where is previously free of the disease. Although some citrus areas are free of the spread of CTV, but the threat continues for the areas having fewer species of aphids (Zhou 1997).

HOST RANGE AND SYMPTOMS INDUCED BY CTV

CTV affects almost all species, varieties, hybrids of genera *Citrus* and some species of *Fortunella* and also experimentally inoculated citrus relatives of the genera *Aegle*, *Aeglopsis*, *Afraegle*, *Atalantia*, *Citropsis*, *Clausena*, *Eremocitrus*, *Hesperthusa*, *Merrillia*, *Microcitrus*, *Pamburus*, *Pleiospermium*, and *Swinglea* (Timmer *et al.* 2000). The host in the non-citrus species *Passiflora gracilis* and *P. coerulea* using aphid vectors (Roistacher *et al.* 1988), and experimental infection in *Nicotiana benthamiana* (Gowda *et al.* 2005). Resistance to CTV isolates varies considerably and has been observed in *Poncirus trifoliata* (L.) Raf. (Rai 2006) and resistance to specific CTV strains has also been observed in Meiwa kumquat (*F. crassifolia* Swing), some pummelos and sour oranges (Asíns *et al.* 2004). CTV replication has been observed in protoplasts of trifoliate orange, pummelo or sour orange, suggesting that only CTV movement is impaired in these species (Albiach-Martí *et al.* 2004).

CTV causes different disease syndromes on citrus plants depending on the virus strain, the variety of citrus and the scion rootstock combination (Moreno *et al.* 2008). Different CTV strains, generally referred to as seedling-yellows (CTV-SY), tristeza (CTV-T), stem-pitting (CTV-SP), and a mild type, have been widespread for many years. Any of these strains may exist in a citrus plant or they may occur together as a complex.

Quick-decline or tristeza disease

Sweet orange, mandarin (including Satsuma and Ponkan), Tankan, Iyo, Tangor, many varieties of tangelo and grapefruit are affected by this disease when grown on sour orange, pummelo or lemon rootstock (but not on rough lemon rootstock). The causal virus is either CTV-SY or CTV-T. When the adult tree is affected by such a combination, it turns yellowing and wilting rapidly and dies within a few years. If the tree is grafted onto resistant rootstock such as trifoliate orange or mandarin, it recovers immediately after grafting (Fraser 1952).

Seedling-yellows disease

Seedlings of self-rooted trees of sour orange, Natsudaidai, lemon, Buntan, and grapefruit are affected by seedling-yellows disease after being infected with distinctive phenotype of some isolates of CTV (Albiach-

Marti *et al.* 2010). These trees become yellow, die back and stunt and sometimes a quite cessation of growth of sour orange, grapefruit or lemon seedlings takes place (Fraser 1952). The physiological mechanism associated with the induction of SY symptoms has not been understood yet. The SY reaction may sometimes be transient. If the affected trees are grafted onto a resistant rootstock, they could be recovered soon. Although it is not economically important but it can be assayed in the glass-house much easily as compared to stem-pitting and decline.

Stem-pitting disease

Most varieties of citrus are affected by stem-pitting disease, even if they are grafted onto a rootstock resistant to tristeza. Although few varieties of mandarin such as Satsuma and Ponkan are resistant to CTV but most of the citrus species such as grapefruit, sweet orange and Buntan and citrus relatives, tangelo, tangor, Iyo, Yuzu, and Natsudaidai are all susceptible. Stem-pitting results from abnormal vascular differentiation, and when the disease is severe, plants develop a large number of pits on both their trunks and their stems. Affected trees become dwarfed and show less vigor (Moreno *et al.* 2008) and occasionally die back. As a result, although there is profuse flowering, the trees bear only poor crops of small sized or irregularly shaped fruits. The severe strain of CTV-SP induced rind-oil spots, or brown spots with gumming on the fruits of some cultivars.

TRANSMISSION AND EPIDEMIOLOGY

Propagation of virus-infected buds is the cause of dispersal of CTV into new areas, while the aphid vector is responsible for local spread. The virus has also been experimentally transmitted to healthy plants by dodder (*Cuscuta subinclusa*) (Weathers and Hartung 1964) and by stem-slash inoculation with partially purified extracts (Garnsey *et al.* 1977) but these procedures are epidemiologically unimportant. CTV isolates have been described as differing in the symptoms induced in the field (da Graça *et al.* 1984) in the reaction induced on indicator plants and in aphid transmissibility (Ballester-Olmos *et al.* 1988). These biological differences may affect the epidemiology of the disease and the damage produced by CTV in different citrus-growing areas. Four aphid species (*Aphis gossypii*, the cotton or melon aphid; *A. spiraecola*, the spirea aphid; *Toxoptera aurantii*, the black citrus aphid; and *Toxoptera citricida*, the brown citrus aphid) have been associated with the natural spread of CTV (Yokomi *et al.* 1994; Rocha-Peña *et al.* 1995). The most efficient vector of CTV worldwide is *T. citricida* (Rocha-Peña *et al.* 1995). The transmissibility of CTV isolates by *T. citricida* is 25 times higher than that of *A. gossypii*.

T. citricida is found in Asia, Australia, Africa, Central and South America, and different Caribbean countries (Rocha-Peña *et al.* 1995; Halbert *et al.* 2004), which is also a serious pest of citrus while both feeding and breeding normally taking place on citrus (Roistacher 1991). CTV was first demonstrated as being aphid transmitted by using hundreds of aphids per plant to transmit the pathogen (Meneghini 1946). *T. citricida* transmission of CTV was reported to have no latent period, but with acquisition and inoculation periods being at least 30 min (Bar-Joseph *et al.* 1989). Also there have been some reports to record the acquisition and inoculation periods of CTV by *T. citricida* being in seconds (Retuerma and Price 1972). Some authors recognize the semi-persistent nature of CTV aphid transmission and additionally classify this transmission as bimodal (Chalfant and Chapman 1962). In bimodal transmission, virus acquisition can cluster around two periods, a short time period and a relatively long time period, and there is generally no change no matter the aphids are pre-acquisitionally fasted or not (Lim and Hagedorn 1977). Variable single aphid transmission rates for *T. citricida* have also been recorded, for instance, up to 25% (Yokomi *et al.* 1994), 0-55% (Broadbent *et al.* 1996), and 16.5-18.4% (Tsai *et al.* 2000). CTV isolates varied in their ability to be transmitted experimentally by the *T. citricida* (Yokomi *et al.* 1994).

ELISA was used to detect CTV (EPPO 2004). A few reports demonstrated the detection of viruses in aphid vectors using the most sensitive PCR-based assays (Cambra *et al.* 2006) either by using RNA purification and RT-PCR (Mehta *et al.* 1997) or by RT-nested PCR (Olmos *et al.* 1999), a multiplex real-time PCR assay (Ananthakrishnan *et al.* 2010). A number of stud-

ies quantitatively estimated the number of viral targets in single aphids (Fabre *et al.* 2003). The spatial and temporal spread of CTV had also been studied in some citrus growing countries (Gottwald *et al.* 1999). These studies showed that the spread of CTV depended on the presence of *T. citricida* or *A. gossypii* as the predominant vector species. In areas where *A. gossypii* was predominant, CTV incidence increased from 5 to 95% in 8-15 years following a stair-step line and infected trees showed limited aggregation, and it was shown that new infections were not related with existing infected trees. Meanwhile, in areas where *T. citricida* was predominant, the same disease rate often occurred in only 2-4 years with a rapid and essentially consecutive increase, and aggregates of infected trees were common because the immediately spread to the trees adjacent to existing infections was frequent. The biology and feeding habits of these vector species perhaps were the causes for these distinct spread patterns (Gottwald *et al.* 1996).

MOLECULAR CHARACTERISTICS OF CTV

CTV, a member of the Closteroviridae (Bar-Joseph *et al.* 1972), genus *Closterovirus*, has a ~20 kb single-stranded, positive sense RNA genome (Karasev *et al.* 1995). This virus genome resides in a single RNA molecule, which produces an unusually large number of less-than-full-length viral RNAs during replication. There are ten genes in the 3′-portion of the genome that are expressed *via* 3′ coterminal sg mRNAs (Karasev *et al.* 1995). Each of the mRNAs produces two additional less-than-full-length RNAs: a negative-stranded RNA with sequence complementary to the sg mRNA and a 5′-terminal positive-stranded sgRNA produced by termination near the controller element upstream of the start of the mRNA, apparently during genomic RNA synthesis (Gowda *et al.* 2001). Hence, CTV produces 30 sgRNAs associated with its ten 3′-terminal genes. The most unusual sgRNAs are two small 5′-coterminal positive-stranded RNAs which have been referred to as 'low molecular weight tristeza RNAs' (LMT) (Che *et al.* 2001). LMT1 and LMT2 RNAs are ~750 and 650 nt respectively (Gowda *et al.* 2003). Both accumulate to high amounts at molar levels higher than that of the virion RNA. LMT1 RNA, which is produced during replication by termination upstream of a previously unknown controller element that produces only minute amounts of a 3′-terminal mRNA-like sgRNA, but without known function (Ayllon *et al.* 2004). The mechanism of production of the smaller LMT2 has been unknown.

The CTV genome consists of 12 open reading frames (ORFs) and potentially encoding at least 19 protein products (Karasev *et al.* 1995). The CTV virons are polarly coated with two separate coat proteins (CPs) p25 and p27, elaborated as major and minor (ca. 3%) CPs, respectively (Febres *et al.* 1996). The minor CP is associated with small amount of two other proteins p65, a homolog of cellular heat shock protein of the 70 kDa family (HSP7O), and a large protein, p61. The 12 ORFs of CTV are expressed through different number of ways including, proteolytic processing of polyprotein, translational frame shifting and up to 32 different 5′- and 3′-subgenomic RNAs (Che *et al.* 2003). The two mechanisms are used to show the protein encoded by the 5′-half of the genome which encodes ORFs 1a and 1b. A large, ~400 kDa polyprotein encoded by ORF1a is proteoltically processed by virus encoded proteases (Karasev *et al.* 1995). ORF 1b is translated by a +1 frame-shift. The second mechanism is used to express the 3′-coterminal ORFs 2 to 11.

From the early genomic characterization of CTV, it is obvious that defective RNAs (dRNAs) are with almost all known CTV isolates. Most of the CTV dRNAs consist of the two genomic termini with extensive internal deletion. CTV isolates have multiple defective RNAs with various large sizes (Zhou *et al.* 1997; Ayllón *et al.* 1999b; Che *et al.* 2003). Recently CTV dRNAs were categorized in six classes (Batuma *et al.* 2004). Different factors contribute to the biological diversity of CTV isolates, such as genetic variation following super-infection with multiple isolates, homologous RNA recombination between sequence variants, the presence of defective RNAs and top working to new varieties (Ayllón *et al.* 1999a, 2006; Roy and Brlansky 2004; Vives *et al.* 2005).

The 5′ half of the genome consists of two ORFs encoding protein associated with viral replication. ORF 1a encodes a large, ~400 kDa polyprotein, that includes two papain-like protease domain, a methyltransferase-like domain and a helicase-like domain. The ORF 1b encodes an RNA dependant RNA polymerase-like do-

main (Satyanarayana *et al.* 1999). The 3´ half of the genome encodes ten genes that are not required for replication in protoplasts (Ayllón *et al.* 2003) with five gene-blocks. The five gene-block is unique to closteroviruses, and encodes a small, 6 kDa hydrophobic protein (ORF3), a 65 kDa cellular heat-shock protein homolog (HSP70h, ORF4), a 61 kDa protein (ORF5), and a tendem pair of structural proteins, a 27 kDa capsid protein (CPm. ORF6) duplicate followed by the 25 kDa (CP ORF7) (Pappu *et al.* 1994; Karasev *et al.* 1995). The small p6 is single-span transmembrane protein not required for virus replication or assembly, which exists in ER and functions in *Beet yellow virus* (BYV), another member of *Closterovirus*, for cell to cell movement (Peremysolve *et al.* 2004). p65 is the homologous of HSP70 heat-shock proteins which together with p61 and two capsid proteins are required for virion assembly (Satyanarayana *et al.* 2004). Protein p20 accumulates in amorphous inclusion bodies of CTV infected cells (Gowda *et al.* 2000). The product of 3´-most ORF (ORF11), p23, is a multifunctional protein with no homologue in other members of *Closterovirus*, that: (i) binds RNA molecule in non-sequence specific manner (Lopez *et al.* 2000); (ii) contains a zinc finger domain that regulates the synthesis of plus- and minus-strand molecules and controls the accumulation of plus strand RNA during replication (Satyanarayana *et al.* 2002); (iii) is an inducer of CTV like symptoms in transgenic *C. aurantifolia* plant (Ghorbel *et al.* 2001); and (iv) is a potent suppressor of intracellular RNA silencing in *Nicotiana tabacum* and *N. banthamiana* (Lu *et al.* 2004). The *p33*, *p18* and *p13* genes are involved in infection and movement in some hosts (Tatineni *et al.* 2008).

The complete nucleotide sequence of CTV has been determined in at least nine distinct isolates (Karasev *et al.* 1995; Albiach-Martí *et al.* 2000; Suastika *et al.* 2001; Vives *et al.* 2005; Ruiz-Ruiz *et al.* 2006). Phylogenetic analysis of the complete sequences reported for nine CTV isolates revealed three main clusters that included (i) the severe SP isolates, T318A from Spain (Ruiz-Ruiz *et al.* 2006), SY568R from California (Vives *et al.* 2005), NuagA from Japan (AB046398) (Suastika *et al.* 2001), and VT from Israel (Mawassi *et al.* 1996); (ii) the mild isolates, T30 from Florida (Albiach-Martí *et al.* 2000) and T385 from Spain; and (iii) T36 from Florida (Karasev *et al.* 1995), Qaha from Egypt (AY340974) and a Mexican isolate (DQ272579). Within-group nucleotide identities were over 97.5%, whereas the lowest identity (75.6%) was between VT and Qaha.

DIAGNOSIS OF CTV

CTV isolates vary in their pathogenicity and also contain various genomic virus variants (Zhou *et al.* 2007) that can be detected by aphids or graft transmission to different citrus host species. The sub-isolates segregated in this way can be differentiated by pathogenicity tests in different hosts, by dsRNA patterns (Moreno *et al.* 1993) or by serologically using specific monoclonal antibodies (Permar e*t al.* 1990; Cambra *et al.* 1993). It has been demonstrated that the haplotype distribution of two CTV genes can be altered after host change or aphid transmission (Ayllón *et al.* 1999). Molecular hybridizations and single-strand conformation polymorphisms analysis of the coat protein gene (Rubio *et al.* 1996) have been used to differentiate the Mediterranean CTV isolates. The best diagnosis method for CTV is to graft-inoculate indicator seedlings of Mexican lime and observe them for vein-clearing, leaf cupping, and stem-pitting (Roistacher 1991). Electron and light microscopy can be used to identify CTV particles and inclusions, but DAS-ELISA (Cambra *et al.* 1979) revolutionised the diagnosis for testing a large number of samples during surveys of large citrus areas for CTV control in nurseries and for epidemiological studies.

The production of monoclonal antibodies specific to CTV (Permar *et al.* 1990) and others reported by (Nikolaeva *et al.* 1996) solved the problems of specificity and increased sensitivity of ELISA tests. A mixture of two monoclonal antibodies (3DF1 and 3CA5) or their recombinant versions (Terrada *et al.* 2000) can recognise all CTV isolates tested from different international collections. A detailed description and characterization of these monoclonal antibodies has been summarised (Cambra *et al.* 2000a). Tissue print-ELISA (Cambra *et al.* 2000b) for CTV detection allowed the sensitive indexing of thousands of samples simply and without the need to prepare extracts. A number of di-

agnostic procedures based on specific detection of viral RNA were developed, including molecular hybridization with cDNA or cRNA probes (Barbarossa and Savino 2006). PCR-based assays have been developed and modified (Zhou *et al*. 2001) based on immunocapture (Nolasco *et al*. 1993) or print or squash capture (Cambra *et al*. 2000c). A simple procedure has been described to perform nested-PCR in a single closed tube (Olmos *et al*. 1999) which allowed CTV detection in single aphids and in plant tissues. A co-operational PCR system (Co-PCR) (Olmos *et al*. 2002) has been described, which supplying similar sensitivity to nested PCR. Real-time RT-PCR protocol is more sensitive and allows the detection and quantification of genomic RNA copies in infected citrus tissues or in viruliferous aphids (Saponari *et al*. 2008). qPCR also is becoming more and more useful as a method for gene expression analysis (Vaudano *et al*. 2009). Through BD-PCR (bi-directional reverse transcription-polymerase chain reaction) analysis, a 392-bp fragment specific for the mild strains was amplified and a 320-bp fragment specific for the severe strains was produced (Jiang *et al*. 2008). The RFLP (restriction fragment length polymorphism) analysis for RT-PCR products of the CP gene with restriction enzyme *Hin*f I identified seven groups (Zhou *et al*. 2007; Jiang *et al*. 2008). RT-PCR amplification patterns with primer set specific for several CTV genotypes (Hilf *et al*. 2005) for 5´-UTR sequence types I, II and III (Ruiz-Ruiz *et al*. 2006) or for three groups of isolates differing by their p23 sequence (Sambade *et al*. 2003). Single-strand conformation polymorphism (SSCP) analysis of different gRNA regions (Sambade *et al*. 2007) has been used to characterize the population structure of CTV isolates and select specific variants for sequencing, thus allowing estimates of the genetic diversity within and between isolates (Ayllón *et al*. 2006). The phylogenetic analysis of p23 showed a high intra-isolate sequence variability suggesting that re-infections could contribute to the observed variability and that the host can play an important role in the selection of the sequence variants present in these isolates (Iglesias *et al*. 2008). Polymorphism analyses of *p23*, *p25* and *p27* genes showed that most isolates contained high intra-isolate variability (Iglesias *et al*. 2008).

STATUS OF CTV IN PAKISTAN

Pakistan is generally considered among the top 10 leading citrus-growing countries of the world both in production and quality. The growing area of citrus in Pakistan is about 193 211 ha with an annual production of 2 459 500 tons (Anonymous 2008). It contributes 2% of citrus fruit to the world's production and earns a major source of foreign exchange for the country. Citrus is grown in all four provinces of Pakistan, but Punjab contributes almost 97% of the production of the country. Generally the growing area and production for different kinds of fruits and particularly for citrus have been increasing since the 1960's due to the increasing demand in the domestic and foreign markets (Khan 1992). In Pakistan fungal and bacterial diseases of citrus have been documented since 1920 through different sources, however virus and virus-like diseases infecting different citrus species could not receive due attention for a long time because of the lack of proper facilities for the detection and characterization (Mughal 2004). Tristeza is a devastating disease in the citrus groves of Pakistan. In Pakistan only limited numbers of surveys have been made to test the presence of the disease in citrus orchards. Survey was made for citrus virus and virus-like diseases in the N.W.F.P and Punjab provinces. Along with other diseases tristeza was detected only in a few trees and confirmed by ELISA and electron microscopy (EM) (Catara *et al*. 1988).

Investigation by EM, threadlike particles of CTV were found in phloem tissues of the columella. CTV was also confirmed by ELISA tests (Grimaldi and Catara 1989). Again a survey was carried out and more than fifty orchards and ten nurseries were sampled in different areas of the Punjab. ELISA tests and EM observations showed that CTV was present in the varieties in different districts. Mosambi was the most affected variety (7 positive out of 35) among the Mosambi, Bloodred and Pineapple sweet orange (Catara *et al*. 1991). Anwar and Mirza (1992) conducted a survey in 14 localities and in five districts, *viz*., Sahiwal, Sargodha, Faisalabad, Lahore, and Sheikhupora, and confirmed the prevalence of CTV by ELISA test with the highest infection (18.8%) in Sahiwal, followed by Sargodha (13.20%) and Faisalabad (13.13%), while no infection was found in Lahore district. In NWFP, symptoms of

vein-clearing and chlorosis were observed in young leaves of *C. aurantium*, *C. limon cv*. Eureka and *C. sinensis* by grafting and mechanical inoculation (Arif *et al*. 2005). Extensive surveys of the major citrus groves in Punjab and N.W.F.P and ELISA tests showed that the CTV incidence in Bhalwal and the Punjab were 44.61 and 48.46%, and in Mardan and N.W.F.P were 37.39 and 40.86%, respectively in 2006-2007 (Iftikhar *et al*. 2009).

CONTROL STRATEGIES FOR CTV

According to the incidence of CTV, strategies to control CTV vary depending on the virus strains and citrus varieties in each particular region (Garnsey *et al.* 1998). Quarantine and bud wood certification programs are useful measures to prevent introduction of CTV into countries where CTV does not exist yet. In citrus groves where CTV incidence is low, the disease can be suppressed by eradication or suppression programs (Gottwald *et al.* 2002). And in the CTV endemic regions CTV tolerant rootstocks, mild strain cross protection and genetically engineered resistance combined with certification programs are the potential ways to deal with the problems (Lee and Rocha-Pena 1992).

Mild strain cross protection is the only best available management method that can be applied to control SP with mild CTV strain where disease is impossible to control by eradication or suppression. This technique has been widely used to control CTV on large scale in commercial citrus plantations, especially with Pera sweet orange in Brazil (Costa and Müller 1980), grapefruit in Australia (Broadbent *et al.* 1991), South Africa, Japan, and limes in India (Lee and Rocha-Pena 1992). These isolates were collected from old trees of the same cultivar that have been grown for years showing only mild or no symptoms. Cross protection is now being implemented in the CTV endemic regions such as Florida (Lee and Brlansky 1990). But unfortunately, the cross protection strategy had less success in other areas or with other varieties (Broadbent *et al*. 1991), which showing that cross protection perhaps has to depend on the varieties, CTV strains and environmental conditions prevalent in each region. *Citrus* sp. such as *C. reticulata*, *C. volkameriana* and *C. jambhiri* (Rangpur lime) are used as root stocks and somehow are tolerant to QD-inducing CTV isolates and some hybrid rootstocks including citranges (*C. sinensis*×*P. trifoliata*) and citrumelos (*C. paradisi*×*P. trifoliata*) are also being used as CTV tolerant rootstocks to control CTV in some citrus growing areas. The presence of other economically important diseases such as citrus blight, viroids, and undesirable horticultural practices limit the usefulness of these rootstocks (Garnsey *et al.* 1987). Moreover, some CTV isolates induce SP symptoms in the scions regardless of the tolerance of their rootstocks (Bar-Joseph *et al*. 1989). Hence, these rootstocks do not give control against CTV-SP isolates where these isolates are widespread.

Genetic engineering gives the specific trait of transgenic plants by incorporating a specific gene into the plants genome without changing the other desirable characteristics. Recent advances in molecular biology and breeding to incorporate resistance genes in commercial varieties have given best results to tackle the problem of crop losses due to pathogens. Plant transformation techniques have opened new vistas and possibilities for the development of sources of virus resistance compared with conventional breeding methods. However, different and complex genetic characteristics of reproductive citrus biology along with their larger plant size have put a great check on genetic improvement through conventional breeding. The first transgenic strategy concept in which a complete or partial gene is introduced into plant to obtain a specific resistance was proposed by (Sanford and Johnson 1985) as pathogen-derived resistance (PDR). PDR for a plant virus was first performed in 1986 (Powell-Abel *et al.* 1986) by introducing the CP gene of *Tobacco mosaic virus* (TMV) into transgenic tobacco plant which ultimately showed resistance to TMV infection and PDR to CTV was for the first time confirmed by the incorporation of the *CP* gene of CTV strain in Mexican lime (Domínguez *et al.* 2002). This concept of PDR has been confirmed in several plant-virus systems (Dasgupta *et al.* 2003). This strategy used in the transformation of other citrus hosts could not give clear or best results (Febres *et al.* 2003). Viral sequences other than CP genes have been explored to engineer PDR to plant viruses. Non-coding sequences from the 5´ and 3´ UTR changed viral genomes of plants as well as satellite RNAs and D-RNAs to produce transgenic plants

resistant to viruses (Nelson *et al*. 1993; Zaccomer *et al*. 1993). Even though the results had some variation with different plant-virus systems, the transformation of non-structural genes in transgenic plants is a promising strategy for developing virus resistance, especially for movement protein and replication associated proteins such as RdRp (Pappu *et al*. 1995). Constructs derived from the CTV 3´-UTR were used to transform sweet orange protoplasts and grapefruit plants but conclusive results on protection at the whole plant level were not reported (Febres *et al.* 2003). Transgenic limes showing the *p23* gene from a severe or from a mild CTV isolate displayed leaf symptoms of similar intensity, which was associated with the accumulation level of the p23 protein (Fagoaga *et al.* 2005). It is clear evidence indicating that disease induction in the host may not be a side-effect of silencing suppression but a consequence of disruption of the miRNA metabolism (Lewsey *et al.* 2007). In short, these results indicate that p23 is an important CTV pathogenicity determinant that interferes with plant development specifically in *Citrus* species and relatives (Fagoaga *et al.* 2005). Transgenic p23UI-*N. benthamiana* were resistant to infection with a viral vector made of *Grapevine virus A* (GVA)+p23U (GVA-p23U), as indicated by the absence of the chimeric virus from inoculated plants. Inoculation of transgenic p23UI Alemow plants with CTV resulted in delayed appearance of symptoms in 9 out of the 70 transgenic plants. However, none of the plants showed durable resistance, as indicated by the obtaining of similar Northern hybridization signals from both transgenic and non-transgenic citrus plants (Batuman *et al.* 2006). Superinfetion exclusion or homologous intereference a phenomenon in which a primary viral infection prevents a secondary infection with same or closely related trait (Svetlana *et al*. 2010) showed that superinfection exclusion of CTV occurred only between the isolates same strain and with different strain.

CONCLUSION AND FUTURE CHALLENGES

Tristeza epidemic is still going on in many citrus regions including Pakistan although 70 years have passed away since its first epidemic. Tristeza decline may cause more losses in future and even destroy the citrus industry of some countries. Dispersal of tristeza induced diseases in Pakistan is increasing very rapidly, which may be able to destroy the citrus industry of Pakistan as well as that of the neighboring countries. While some management strategies may eventually tackle the problem and restore the citrus production, such as replacement of declining trees with new trees on tristeza tolerant rootstocks, resistance genes, cross protection, identification of pathogenicity determinants for different disease syndromes. Citrus production can be increased through nurseries running on a scientific and professional basis. Certified citrus nurseries are needed to solve the problem caused by CTV. Better understanding of the relationship among CTV isolates, host plants and vectors should be strengthened, which has been absolutely limited in Pakistan. The combination of using certified budwood programs plus MSCP strategy is certainly the best way to control the losses induced by CTV.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30471205), the Special Fund for Agroscientific Research in the Public Interest, China (200903004-06), the Program for Changjiang Scholars and Innovative Research Team in University, China (PCSIRT, IRT0976), and the Chinese Scholarship Council, China (CSC). Special thanks go to Prof. Li Zhongan, Southwest University, China, for his kind help.

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(Managing editor ZHANG Juan)