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Viruses, greening bacterium and viroids associated with citrus (*Citrus* species) decline in India

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ABSTRACT

Three viruses, viz citrus tristeza (CTV), ring-spot (CRSV) and citrus mosaic badnavirus (CMBV), the greening bacterium (GB) recently classified *Liberobacter asiaticum* and the viroid infections, were identified as the major pathogens associated with citrus (*Citrus* spp) decline in India. The information on characterization and development of diagnostic reagents for their quick detection provided new avenues for their management. Variation in strains of pathogens and biotypes in the vectors is helpful in the management of vector-borne viruses through cross-protection. CRSV and CMBV are newly discovered viruses and their incidence in Indian citrus is 10 – 70%. Both these viruses are transmitted through bud and hence can be effectively managed by bud selection. The monoclonal antibodies developed for CTV and GB have been used for pathogen-specific detection in enzyme-linked immunosorbent assay (ELISA). However, CMBV and GB have also been detected by nucleic acid probes in DNA–DNA hybridization, whereas CRSV is detected only by serological methods and viroids by polyacrylamide gel electrophoresis and specific nucleic acid probes. These methods will help develop sound indexing programmes for budwood certification. Evidence shows that the earlier budwood certification and cross-protection programmes in India failed due to the lack of proper detection techniques, which are now available and can help in developing effective management practices.

Key words : *Citrus* spp, citrus decline, viruses, greening bacterium, viroids, detection technique, association of pathogens

Decline of citrus (*Citrus* spp) in India is a century-old problem, caused by both biotic and abiotic stresses. Virus or virus-like infections in citrus were not well understood in the past. Therefore any protection programme initiated in India did not yield desirable results. Virus infections are now known to be a major constraint in citrus production all over the world. Removal of these infections from the planting material greatly improved the tree health and its yield. In India useful information has been developed on tristeza, ring-spot and mosaic viruses during the last decade. The infection by greening bacterium (*Liberobacter asiaticum*) and viroids to *Citrus* spp has also been worked out in relation to decline. In mid-1960s, kinnow mandarin (*Citrus reticulata* Blanco) ('King' x 'Willow Mandarin') was introduced in India from California. Individual mother tree of the progeny being maintained at Abohar (Punjab) when examined for ring-spot disease revealed 100% contamination with this virus. It indicated that the original planting material introduced from California might be contaminated with ring-spot virus (CRSV) or the virus might have existed in India on indigenous *Citrus* spp and moved to the newly introduced kinnow mandarin. Citrus ring-spot disease has now spread to indigenous cultivars like malta, mosambi and sathgudi sweet oranges [*Citrus sinensis* (L.) Osbeck] both in north and south India. This article reports the association of these pathogens with citrus decline.

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STATUS OF RESEARCH ON CITRUS VIROLOGY IN INDIA

Tristeza and greening diseases were considered to be the main cause of decline of citrus in India (Capoor 1963, Raychaudhuri *et al.* 1977, Ahlawat and Raychaudhuri 1988). This conclusion was based on limited indexing on indicator hosts, as no other means of virus detection were available. During the last 2 decades it was realized that biological indexing has its own limitations, and hence attention was diverted toward development of newer methods of indexing for facilitating large-scale indexing. During this process several new virus and virus-like disorders were discovered in various parts of the world, including India. Researches were

strengthened to characterize the viruses infecting citrus and novel techniques and diagnostic reagents were developed for effective indexing programmes. These developments changed the old outlook and generated new and useful information. The techniques generated during recent times proved useful for the improvement of citrus crop.

In India several viral, viroid and phytoplasmal disorders of citrus were investigated during the last 20 years (Reddy *et al.* 1974, Mali *et al.* 1975, 1976, Murthi and Reddy 1975, Ahlawat and Sardar 1976, Yora *et al.* 1977, Ahlawat *et al.* 1979, 1984, 1985, 1993, 1996, Sharma and Pandey 1983, Ahlawat 1989, Byadgi *et al.* 1993, Ramachandran *et al.* 1993), but the etiology of most of these diseases could not be established. Among the several such disorders, 5 diseases, viz tristeza (closterovirus), ring-spot (capillovirus), mosaic (badnavirus), exocortis viroid and greening (*Liberobacter asiaticum*), were identified as potential pathogenic diseases of the decline complex. The diagnostic reagents were developed for their quick and reliable detection

Tristeza

Brown (1920) recorded the failure of malta sweet orange [*Citrus sinensis* (L.) Osbeck] on sour orange (*C. aurantium* L.) rootstock, providing evidence of flourishing tristeza disease in India. Estimates indicate that tristeza destroyed 25 million trees in south Africa, 6 million in Venezuela, 3 million in California and 1 million in India. In view of the gravity of the tristeza disease, attempts were made to manage it by cross-protection in several countries including India at Tirupati and Bangalore (Balaraman and Ramakrishnan 1977). However, conflicting reports were obtained regarding tree protection from severe strains of CTV in these experiments. The serological studies conducted with monoclonal antibodies (MAbs) in enzyme-linked immunosorbent assay (ELISA) showed that all the trees at Bangalore and Tirupati experiments carried severe strains irrespective of their inoculation with mild, severe or chellange (mild followed by severe) strains. The uninoculated control trees at Bangalore were also contaminated with severe strain of CTV but the uninoculated ones were healthy at Tirupati. A comparison of the spread of CTV in Bangalore region with that in Tirupati (Chakraborty *et al.* 1993) indicated the occurrence of biotypes in CTV vector (*Toxoptera citricida* Kirk.).

Flexuous thread-like virions, 10–12 nm x 2 000 nm, are constantly associated with tristeza-infected citrus plants and are believed to be the cause of the disease. The virus is limited to phloem and belongs to closterovirus group. It has single-stranded positive-sense RNA of 20 000 nucleotides as its genome, consisting of a single-type coat protein with Mr 26 000. The CTV coat-protein gene (CPG) was selectively identified in polymerase chain reaction (PCR) and was cloned (Manjunath *et al.* 1993). The coding region of CPG was expressed in *Escherichia coli* (Migula) Castellani & Chalmers. In addition to biological indexing on kaghzi lime [*C. aurantifolia* (Christm.) Swingle], diagnostic reagents based on CTV-coat protein and genomic RNA have now been developed for quick indexing of CTV. Strain-specific monoclonal antibodies (MAbs) such as CTV-MCA 13 are developed to identify protective mild strains for the management of CTV by cross-protection against severe or disastrous strains.

Ring-spot

This is a newly discovered disease in India (Byadgi *et al.* 1993, Byadgi and Ahlawat 1995). Citrus ring-spot virus (CRSV) is characterized as ssRNA virus with 29 KDa coat protein, which is a member of capillovirus group (Byadgi *et al.* 1993). Two types of virions, measuring 640 nm x 15 nm and 690 nm x 9 nm, were associated with ring-spot disease in addition to tubule-like inclusions. The virions of size 690 nm x 9 nm and tubules have been considered a by-product of the capillovirus (Byadgi *et al.* 1993) and used in virus detection.

Except bud transmission, no other mode of natural spread of CRSV could be established so far. Recently CRSV virions were observed in immunosorbent electron microscopy (ISEM) from pollens of contaminated kinnow trees, indicating transmission of the virus through pollen. Byadgi and Ahlawat (1995) reported that CRSV could infect most of the commercial citrus cultivars and rootstocks used in India. Ahlawat *et al.* (1995) developed diagnostic reagents and techniques for quick detection of CRSV in the planting material.

A flexuous rod-shaped virus is associated with most of the ring-spot isolates studied in various parts of the world. Isolates from India and California had a 29 KDa protein, whereas others had that of 48–50 KDa. However, virions associated with Indian isolates belonging to capillovirus group are distinct from viruses of ring-spot reported elsewhere.

Mosaic

The mosaic disease has been reported in sathgudi and chini sweet oranges [*C. sinensis*] from south India and in pummelo [*C. decumana* L.; syn *C. grandis* (L.) Osbeck] from north-eastern India (Ahlawat *et al.* 1985, Reddy and Murti 1985). Its incidence is 10–70%, causing serious loss in orchards, which are even abandoned. Its presence in nurseries indicates its spread through contaminated budwood.

Mosaic disease is transmitted by bud graft and by mechanical inoculation from among citrus species. Bacilliform virions measuring 130 nm x 30 nm were observed under electron microscope (Ahlawat *et al.* 1993, 1996).

Citrus mosaic virus has been identified as a member of badnavirus group, as it reacted specifically to antisera of many badnaviruses in immunosorbent electron microscopy (ISEM) tests. The genomic nucleic acid (DNA) of the virus was also amplified in polymerase chain reaction (PCR) using badnavirus-specific oligonucleotide primers (Ahlawat *et al.* 1996). Based on these studies this virus is designated citrus mosaic badnavirus (CMBV), which is the first report.

Greening

Fraser *et al.* (1966) reported the existence of greening disease in India, transmitted by oriental psyllid vector (*Diaphorina citri* Kuway). Greening was reported from different parts of India mainly on the basis of symptomatology, particularly zinc-deficiency symptoms on leaves. However, leaf mottle, a typical symptom of greening, was overlooked.

Until the report of Garnier *et al.* (1984 a, b) that greening pathogen was a phloem-restricted gram-negative bacterium, it was considered to be a virus. All these studies were made from a greening isolate from Pune. The greening bacterium has not yet been cultured in cell-free medium, and it is therefore called a 'bacterium-like organism' (BLO). Recently, on the basis of 16s rDNA sequences as well as sequences of the rplKAJL-rpoBC operons to probe in 2.6 and as 1.7 Kb, the BLOs associated with citrus-greening disease have been identified as bacterium and classified as *Liberobacter asiaticum* for Asian greening and that of South African greening as *L. africanum* (Jagouex *et al.* 1995).

Another breakthrough in the study of greening bacterium was its experimental transmission to periwinkle (*Catharanthus roseus* L.) by dodder (*Cuscuta campestris* L.), which helped in the preparation of bacterium-specific detection reagents. Use of monoclonal antibodies (MAbs) and nucleic acid probes developed from the Pune strain of the bacterium (Bove *et al.* 1993, Varma *et al.* 1993) showed variable incidence of greening disease in various states in India (Ahlawat *et al.* 1995, Varma *et al.* 1993). The trees with typical dieback symptoms in Nagpur region were without leaf-mottle and gave negative results for the greening bacterium in electron microscopy and DNA–DNA hybridization using a 2.6 Kb DNA fragment from Pune strain of the greening bacterium, which was cloned and used as a probe. This probe also proved effective in detecting the bacterium in individual *D. citri*, the vector of greening.

Psyllids can acquire greening bacterium (GB) during feeding (Capoor *et al.* 1967). The low concentration of the GB and its erratic distribution in citrus tissue may sometimes account for low percentage of transmission. After acquisition, the GB passes through the intestinal wall and travels to the acini of the salivary glands, from where it is injected to the plants. This is normally a long process (1–3 weeks) for most of the prokaryotes. Except in a few endemic regions for greening like Coorg in Karnataka and Hindupur in Andhra Pradesh, field spread of the GB was observed very slow. It indicated the presence of biotypes in *Diaphorina citri* in India. Adult psyllids fed on a known GB-infected pineapple sweet orange [*C. sinensis*] plant (Bangalore isolate) for different periods were unable to transmit the GB. The GB was also not detected in individual psyllid by DNA–DNA hybridization with 2.6 Kb probe, though it can specifically detect the GB in individual psyllids. It shows that adult psyllids are not capable of acquiring the GB and transmitting it. Therefore either the psyllids acquire the GB at their nymphal stages or there are some biotypes of *D. citri* that alone act as efficient vector. Transmission and hybridization results revealed that biotypes of Indian psyllid (*D. citri*) are less efficient vector than of Malaysian psyllids.

Different serotypes in the GB were determined with the help of MAbs (Ahlawat *et al.* 1995). However, MAbs prepared from Pune strain of the GB also recognized the Malaysian GB, indicating similarity in the GB found in these countries. But the presence of more number of viruliferous psyllids in Malaysia shows distinct biotypes in the vector (*D. citri*).

Among the commercial cultivars, group of sweet oranges [*C. sinensis* (L.) Osbeck] is more sensitive to the GB than the groups of lemons [*C. limon* (L.) Burm. f.] and limes [*C. aurantifolia* (Christm.) Swingle]. Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is tolerant to GB, but it is infected with GB in Coorg region. So far no resistance was found in any *Citrus* species or related genera. Hence the prospects of successful breeding of new scion varieties inheriting workable resistance is still remote. However, the disease symptoms can be suppressed by injecting the affected trees with 500 or 1 000 ppm tetracycline or penicillin under pressure (10 kg^2/cm) (Kapur *et al.* 1992). Regular treatment with these drugs can reduce the loss caused by greening disease.

Viroids

Nariani *et al.* (1968) reported the symptoms of exocortis, a viroid disease, in India but there is no information on its etiological agent. Recently viroids have been identified by return polyacrylamide gel electrophoresis (R-PAGE) in samples collected from various places in India. The R-PAGE analysis revealed the presence of viroids in 2 types of affected trees: one showing typical bark-scaling symptoms of exocortis-type disease, which is transmissible to its indicator host, *C. medica* L., and the other category of trees did not show any bark-scaling effect and the viroid was transmissible to 'Suyo' cucumber (*Cucumis sativus* L.). Two different viroid species were found associated with 2 types of symptoms (Ramachandran *et al.* 1993), ie citrus viroid IIa (Duren-Villa *et al.* 1988) and Indian tomato bunchy-top viroid (ITBTVd) (Mishra *et al.* 1991). The trees affected by these viroid species showed different degrees of decline. Viroid IIa is a common infection to rootstocks like trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and rangpur lime (*C. limonia* Osbeck), which did not prove better rootstock than rough lemon (*C. jambhiri* Lush.) and hence are not popular in India. Recent investigation showed that ITBTVd can infect the trees on rough lemon rootstock without visible symptoms (Ramachandran *et al.* 1993). Therefore further evaluation of different scion combinations on rough lemon roots is necessary to establish the damage caused by these pathogens. Presence of these pathogens in Indian citrus may cause concern in the near future, as they are symptomless, seed-borne and are transmitted by contaminated tools. Recently a

disease showing yellowing and corking of veins of kaghzi lime [*C. aurantifolia* (Christm.) Swingle] showed the presence of a previously unknown viroid.

ADVANCES IN DETECTION OF VIRUS, VIROID AND GREENING BACTERIUM

Tristeza

Field diagnosis of tristeza is very difficult, as no visible symptoms are developed on affected trees except on kaghzi lime (*C. aurantifolia*) and some mandarins (*C. reticulata* Blanco) like coorg orange, where stem pitting is developed. Citrus tristeza virus (CTV) is more harmful to Indian sweet oranges than to other cultivars (Ahlawat *et al.* 1995). Kaghzi lime is used as an indicator host of CTV, but the diagnostic vein-flecking symptoms on this host depend on temperature. No foliar symptoms are developed at temperatures higher than 27°C and hence its biological indexing on large scale is difficult. Indexing by serological methods to analyse more samples at a time is possible by newer techniques like enzyme-linked immunosorbent assay (Ahlawat and Raychaudhuri 1988, Chakraborty *et al.* 1993). Monoclonal antibody MCA-13 is used for differentiating the strains in CTV for cross-protection studies. For higher specific detection of CTV, more techniques like nucleic acid hybridization and polymerase chain reaction (PCR) are being developed in India.

Ring-spot

The rings on mature leaves of *Citrus* spp, which may be 1 to several per leaf with a diameter of 0.2–2.0 mm with green tissue in the centre, are the diagnostic symptoms of the disease. Healthy mosambi sweet orange plants develop typical vein-flecking symptoms on inoculation of scion with ring-spot symptoms. The virions of CRSV both from field and glass-house-infected plants are detected in electron microscope in leaf-dip preparations with negative staining. This virus is also detected at lower concentrations in enzyme-linked immunosorbent assay and also by trapping the virions by immunosorbent electron microscopy.

Mosaic

Bright yellow mottling of leaves especially on sweet oranges (*C. sinensis*) and pummelo [*C. decumana* L.; syn *C. grandis* (L.) Osbeck] is the main diagnostic symptom of the mosaic disease in the field. The bacilliform particles of the virus are detected in electron microscopy and in enzyme-linked immunosorbent assay. The virus has also been detected in polymerase chain reaction (Ahlawat *et al.* 1996), which is a highly reliable detection technique. Recently 2.0 Kb DNA fragment of the virus has been cloned and used for virus-specific detection from field-infected trees.

Greening

Field identification of greening disease is often difficult, because the symptoms on affected trees are sometimes confused with that of zinc and other nutritional deficiencies. Therefore Ahlawat *et al.* (1995) tested citrus trees showing variable symptoms with electron microscopy, enzyme-linked immunosorbent assay and DNA–DNA hybridization, and concluded that mottling of the leaves of affected trees is its main symptom. Unusual colouring of fruits and aborted seed were the additional symptoms. Transmission of the greening bacterium to periwinkle by dodder is a useful technique to identify greening disease. Electron microscopy of ultra-thin sections can detect the GB in sieve tubes of affected leaves. No polyclonal antibodies could be developed for the GB so far, because of its pleomorphic nature and inability to culture in synthetic media. MAbs have however been prepared and used for strain-specific detection (Bove *et al.* 1993, Varma *et al.* 1993, Ahlawat *et al.* 1995). These MAbs can recognize only the strains from which these were prepared; 2.6 Kb nucleic acid probe can detect most GB strains present in India and other Asian countries. It is therefore now possible to detect more strains of GB in enzyme-linked immunosorbent assay, immunofluorescence and DNA–DNA hybridization (Varma *et al.* 1993, Ahlawat *et al.* 1995).

Viroids

Bark scaling in rootstock and stunting of a tree indicate the presence of a viroid infection. But these may be present even without expressing any visible symptom (Ramachandran *et al.* 1993). Viroid infection in trees is detected by indexing on Etrog citron (*C. medica* L.), which develops epinasty of leaves upon inoculation. However, symptomless infection is detected by inoculating 'Suyo' cucumber where symptoms are similar to that of Indian tomato bunchy-top viroid. The viroid species are detected in reverse polyacrylamide gel electrophoresis (R-PAGE) by observing differences in their electrophoretic mobility.

DISTRIBUTION

North-West Zone

The zone comprises Punjab, Haryana, Rajasthan and parts of Gujarat. Citrus ring-spot is the major disease of kinnow mandarin, malta sweet orange and lime in this region. Tristeza and greening diseases although present show negligible spread, because the efficient vector of CTV (*Toxoptera citricidus* Kirk.) and efficient biotypes of *Diaphorina citri*, the vector of greening, are not active in this region.

North-East Zone

It includes Sikkim, Darjeeling district of West Bengal, Assam, Meghalaya, Manipur, Nagaland, Arunachal Pradesh and Tripura. The major citrus plantations in this region are the seedling trees of mandarin and lemon. Tristeza is the major disease, as the vector *T. citricida* is very active. Other localized viral infections like lemon crinkly leaf and leathery leaf, rubbery-wood phytoplasma and greening bacterium are present, but appear to be of limited importance in citrus decline.

Central Zone

It comprises Maharashtra and Madhya Pradesh and is the major citrus - growing belt in India. Greening has been found in a few mandarin trees (*C. reticulata* Blanco) at Amravati compared with a high incidence of the GB in Pune region. Citrus tristeza (CTV) singly or with GB was common in Pune region but not in Vidarbha. Heavy infection of *Phytophthora* spp especially *P. palmivora* Butler causing varying degrees of root-rot, gummosis or crown rot (Lele and Kapoor 1982, Naqvi 1988) is the major factor of dieback in this region.

South Zone

It includes parts of Andhra Pradesh, Karnataka, Tamil Nadu and Kerala. Citrus tristeza (CTV), mosaic (CMBV) and greening (GB) are the major infections in this region. In Kodagu region citrus cultivation is almost abandoned due to infection of greening (GB) and CTV. Sathgudi sweet oranges are declining due to CMBV infection in Andhra Pradesh. Mixed infection of CTV and CMBV and of CRSV or CTV and GB are common.

FUTURE THRUSTS

During the last 50 years the trend has been to plant fewer and fewer varieties concentrated in larger and larger areas of contiguous orchards. These conditions favour rapid spread of virus and other diseases when undisciplined and unsanitary mass-propagation techniques are used to produce nursery trees. Spread of viruses by insect vectors is also favoured in vast-area plantings that approach monoculture. The virus or virus-like diseases limit the yields of Indian citrus and need to be controlled. No practical control measures are available for the infected plant. Avoiding the disease through use of disease-free budwood, reinforced by regulations to limit movement, sale and use of infected budwood or planting stock, are the principal control measures.

The basic knowledge has been generated on important virus and virus-like diseases of Indian *Citrus* spp. Viral infections are the major cause of citrus decline at various locations in India. Therefore production of healthy budwood is important to manage these diseases. Previously it was necessary to grow nucellar clones from seed to maturity to remove such infections, but this method requires 10–15 years to produce usable disease-free budwood for commercial propagation. However, with monoembryonic varieties like pummelo (*C. decumana*) even this method could not be used, because seedlings are all zygotic, ie genetically different from the mother plant. The techniques like heat therapy and shoot-tip micrografting are now available for rapidly obtaining pathogen-free clones even from diseased clones. Such techniques must be used widely in India, especially to eliminate CRSV from kinnow mandarin and CMBV from sathgudi sweet orange.

For healthy budwood production and maintenance of healthy mother trees, sound indexing procedures are essential. Serological detection techniques have been developed for CTV, CMBV, CRSV and GB and molecular detection methods for GB and CMBV. However, more monoclonal antibodies are required to detect strains of CTV and GB on regional basis in India.

In view of the wider distribution and very high incidence of CRSV, it is essential to experimentally establish its natural mode of spread other than bud transmission. Virus-free nucleus material in kinnow mandarin must be obtained through heat therapy and shoot-tip micrografting. Another area of research is to study and identify strains in CRSV by biological and serological techniques, to generate successful cross-protection programmes.

As CMBV is an important virus of citrus in India, it is essential to develop diagnostic reagents for its serological and molecular detection and to develop detection kits for orchardists and nurserymen. Although Murthi and Reddy (1975) reported that citrus mosaic is transmitted by black citrus aphid [*Toxoptera citricida.* (Kirk.)], the report needs confirmation. Most of the badnaviruses are transmitted by mealy bugs (*Planococcus* spp). Therefore field spread of CMBV must be investigated using different mealy bugs and other insect species feeding or visiting citrus.

Very little work has been done on viroids infecting citrus in India, and this aspect needs more work for characterization of various viroid species infecting citrus in India. Nucleic acid probes must be prepared for quick and reliable detection of viroids, so that the effect of these pathogens could be evaluated on different commercial scion varieties on rough lemon rootstock.

These pathogens, however, could not be established in declining citrus trees in Nagpur region, but association of an unknown virus cannot be ruled out. However, the role of greening in the dieback syndrome of Nagpur orange trees (*C. reticulata*) remains to be evaluated. Evidence shows the failure of earlier budwood certification and cross-protection programmes in India due to lack of proper detection techniques, which are now available. Molecular probes for specific detection of CRSV and viroids need to be developed for developing sound indexing programmes on *Citrus* spp in India.

However, the high average yield of citrus depends not only on the use of healthy budwood but also on better disease-tolerant stocks and cultural practices. But unless the orchards are planted with virus-free nursery stock, none of the potentials of the improved practices are likely to be fully realized.

It is necessary to generate information on epidemiology of virus diseases of citrus, which has not been adequately studied. The future breeding strategies for citrus through biotechnological methods must be initiated to develop resistant cultivars.

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