Chapter

Indexing Virus and Virus-Like Diseases of Citrus

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Abstract

Citrus is a highly nutritive and prized fruit crop around the world. It contributes a substantial share in local consumption and exports of a nation to earn a handsome foreign exchange. The production of citrus is under the threat of citrus decline. Different factors are responsible for the citrus decline but virus and virus-like diseases have the major role in this decline. Virus and virus-like diseases alone or in association with other biotic and abiotic factors exist in the citrus orchards. Therefore, indexing of diseases caused by virus and virus-like pathogens is the key factor to manage these citrus diseases. Proper facilities and skilled personnel are the pre-requisite for the diseases indexing procedures. Biological, serological and molecular indexing is sensitive, reliable and durable strategy for managing different citrus virus and virus-like diseases under different conditions. Moreover, indexing of viruses and virus-like pathogens are very important for the production of disease free citrus nurseries. This chapter gives a brief review for the commonly used biological, serological and molecular assays for the detection of citrus virus and virus-like pathogens.

Keywords: citrus, detection, diseases indexing, viruses and virus-like pathogens, graft-transmissible diseases, viroids, RT-PCR, ELISA

1. Introduction

Citrus belongs to family Rutaceae and holds an important position among fruits all around the globe. It is the most cultivated fruit in the world after grapes. Citrus is believed to be originated from southeastern Asian region [1]. Northern hemisphere accounts for about 70% of the total citrus production and approximately 80 citrus species are native to India and other tropical and sub-tropical areas of Asia [2]. Citrus being a perennial fruit tree is usually produced through vegetative propagation of scion on rootstock. Combination and compatibility of scion and rootstock can result in high yielding citrus plants. The United States, China, Brazil and the Mediterranean countries contribute two third of global citrus production and are regarded as major citrus producing countries [3]. Citrus products and by-products provide the basis for local agricultural industries, which generate employment and raise income, and in many cases, this industry constitutes an important source of foreign revenue for developed and developing countries such as Pakistan.

A number of factors and certain conditions are collectively responsible for fluctuations in citrus production. Selection of rootstock, agronomic practices and

management in citrus nurseries and orchards, propagation methods and biotic and abiotic factors contribute their share to some extent in reduced citrus production. Like other commercial crops, number of diseases, insect pests and genetic problems affect the citrus production. Diseases are one of the major limiting factors for the low citrus production and gives a serious threat to citrus industry. These diseases are caused by fungi, prokaryotes, nematodes, viroids, viruses and virus-like pathogens. Among these, viruses and virus-like pathogens play a major role in citrus decline. These pathogens incur varying degree of damages to citrus plants and make their life span shorter, causing low yield and deterioration of quality and ultimately loss of economy which leads towards the citrus decline [4].

Citrus decline is the matrix of all above mentioned factors and conditions. The common diseases, playing an important role in citrus decline are citrus gummosis caused by *Phytophthora* sp. and *Fusarium* sp., citrus canker caused *by Xanthomonas* sp., Huánglóngbìng caused by *Candidatus laberibactor* sp., citrus stubborn caused by *Spiroplasma citri* and one of the most devastating citrus viruses *i.e.* citrus tristeza virus. Citrus viruses play a vital role in its decline by using the prevailing conditions and many other factors as these are bud/graft-transmissible and have systemic infections. A variety of symptoms has been observed regarding the infection of citrus viruses resulting in systemic infection. No viral diseases on citrus was under discussion or the hot issue before 1940 but during and after 60 years, thirty economically important viruses and virus-like diseases of citrus were recognized as a cause of citrus decline in different parts of the world [5–7]. Unfortunately, citrus orchards are short lived and decline within 15 years as against their potential of 50 years or more. This is mainly attributed, among other factors, to the prevalence of graft-transmissible virus and virus-like diseases, faulty nursery operations and poor orchard management. However, most of the problems originate from nurseries.

Therefore, it is the time when citrus nurseries should operate on highly technical and scientific lines and start providing disease-free and certified plants to the growers. In the first instance, nurseries should be registered and indiscriminate multiplication and sale of uncertified citrus plants must end. For this purpose, the most imperative points such as the prevalence and detection of citrus viral diseases, selection of material, production of disease-free material and streamlined screening procedures are highlighted in this bulletin. If the guidelines are properly followed and certified bud-wood becomes available for producing disease-free citrus plants, the problem of citrus decline can be minimized.

1.1 Citrus pathology

Citrus pathology is the study of citrus diseases caused by biotic (pathogens) and abiotic factors. It is now being considered as a major part in the field of plant pathology. Being a major fruit crop in the world, citrus production always remains important for the citrus industry. Physiology, morphology, biochemistry and behavior of the citrus tree towards the prevailing climatic conditions are the key areas to be kept in mind while investigating the citrus diseases. Etiology of citrus diseases and their detection methods help to manage these diseases. A plenty of information regarding the diseases of citrus and their control has been published around the world.

2. Virus and virus-like diseases of citrus

Virus, viroids and virus-like diseases, however, infecting different citrus species could not receive due attention because of the lack of laboratories with proper facilities for their proper identification. These diseases are also known as

'graft-transmissible diseases' (GTDs) and the term used for the casual agents is 'citrus graft-transmissible pathogens' (CGTPS) [8]. These are an emerging threat for citrus industry. Major viruses and virus-like pathogens include citrus tristeza virus (CTV), citrus yellow vein clearing virus (CYVCV), citrus variegation virus (CVV), concave gum, psorosis, cristacortis, ringspot, exocortis, *Cachexia-xyloprosis*, *Candidatus liberibacter asiaticus* and *Spiroplasma citri* [9, 10]. A brief description of these virus and virus-like pathogens is summarized below (**Table 1**).

Although plant pathologists have put their efforts for the identification and management of virus and virus-like diseases of citrus but there are some areas need to be investigated. A comprehensive book has been written by Roistacher in 1991 regarding the detection of virus and virus-like diseases of citrus. These diseases reduce the citrus yield and ultimately result in the loss of low foreign exchange. Diseases caused by viruses and virus-like pathogens are infectious, contagious and devastating due to their systemic nature. They are transmitted through different means in nature; through vegetative propagation, by insect vectors and horticultural tools used for the routine activities in citrus orchards and nurseries. These diseases have a considerable economic importance because of their involvement in

Sr. No.	Citrus disease	Pathogen	Transmission	Incidence %	Host	Importance
1.	Citrus greening disease (CGD) Huánglóngbìng	Bacterium-like organism	Psyllid: Diaphorina citri	20–90	Sweet orange, grapefruit, orange jessamine	Associated with citrus decline
2.	Citrus tristeza virus (CTV)	Closterovirus	Aphid species (Aphis gossypii, Toxoptera citricida)	7–18 Up to 48	Sweet orange, lime, mandarin	Economically important
3.	Gummy bark (GB)	Virus probable	Grafting, mechanical	20–30	Mandarin, sweet orange	-do-
4.	Bud union crease (BUC)	Virus probable	Grafting, mechanical	20–30	Mandarin, sweet orange	-do-
5.	Cristacortis	Virus probable	Not known	10	All citrus species	-do-
6.	Exocortis	Viroid	Mechanical	7–10 Up to 16	Sweet lime	-do-
7.	*Cachexia-xyloporosis*	Viroid	Mechanical	4–10	Mandarin	-do-
8.	Citrus stubborn disease (CSD)	Prokaryote	Leaf hopper (*Neoaliturus haemocops*)	2–7	Sweet orange, grapefruit, periwinkle	-do-
9.	Yellow vein clearing (YVC)	Virus	Grafting, vector not known	2	Lemon, sour orange	Minor importance
10.	Ring spot/ Variegation	Virus	Not known	2–3	Sweet orange	Minor importance

Note: *All diseases are graft-transmissible. No adequate information on vector transmission is available except their identity; viroids problems are favored by warm conditions.*
**The above summarized information is extracted from the work of [10–12].*

Table 1.
Major virus and virus-like diseases of citrus in Pakistan, their transmission and hosts.*

Citrus species	Virus				Viroid		Prokaryote		Virus-like symptoms					PS	DE
	CTV	IVV	RS	YUC	Ex	CX	GR	ST	BS	GB	BU	BP	Misc.		
Sweet orange (Mosambi)	+		+		+	+	+	+		+	+	+	+	+	+
Sweet orange (Mosambi)	+		+		+	+	+	+		+	+	+	+	+	+
Mandarin	+					+	+		+	+	+	+	+	+	+
Sweet lime					+						+	+	+	+	+
Grapefruit								+		+	+	+	+	+	
Lemon	+	+	+	+											
Acid lime	+								+				+		+
Rough lemon		+	+				+							+	
Sour orange	+	+		+									+		
Orange jessamine					+		+			+					

Note: *"+" is the indication of presence of infection on the citrus varieties.*
CTV = Citrus Tristeza Virus, IVV = Infection variegation, RS = Ring spot; Ex = Exocortis, CX = Cachexia xyloporsis, GR = Greening disease, ST = Stubburn Disease, BS = Bark Scaling, GB = Gummy Bark, BU = Bud Union Disease, PS = Psorosis, DE = Decline.
[10, 12].

Table 2.
Citrus species and presence of viruses and virus-like diseases.

the citrus decline [4]. Millions of citrus trees have been died due to CTV. The CGTPS usually have two types of effects either quick decline or long term losses. These diseases are very difficult to control or manage unless or until by the application of integrated management practices. The appropriate diagnosis or indexing method plays an important role for the management of CGTPS [8].

The major symptoms due to virus and virus-like pathogens are vein clearing, bark cracking, yellowing of leaves, leaf dropping, gummosis, mosaic, rugosity, bark scaling, stem pitting, dwarfing, chlorosis and mottling [10, 13]. The virus and virus-like diseases, infecting different citrus species in Pakistan, have been neglected for a long time due to lack of proper facilitations in the research laboratories and skilled personnel for their detection and characterization. A brief description is presented in **Table 2** regarding the citrus species and viruses and virus-like diseases in Pakistan. Indexing facilities are very important for the diagnosis of plant pathogens. Similarly, unlike other pathogens viruses and virus-like pathogens are very sensitive to their indexing through different techniques. Pathogen detection system always played an important role in management of virus and virus-like pathogens. Proper indexing facilities help in the characterization and differentiation of different viruses and their isolates. Management of viruses and virus-like pathogens is only possible when appropriate indexing procedures and facilities are available.

3. Insects as vectors of virus and virus-like pathogens

Insect pests have always been key role players in the direct or indirect transmission of plant pathogens in agricultural and horticultural crops [14–16]. Citrus tristeza, cachexia-xyloporosis, greening or Huánglóngbìng, infectious variegation, vein

enation, yellow vein clearing, exocortis and stubborn are the most conspicuous viral diseases of citrus all over the world including Pakistan [11, 17]. These diseases are usually graft-transmissible and phloem-restricted. Although these diseases along with other fungal, bacterial or mycoplasmic infections of citrus are usually spread through unhealthy mechanical intrusions and by the use of infected uncertified bud, scion or rootstock in plant propagation, many type of sap-feeding insect pests play important role in the transmission of these diseases such as leafhoppers, aphids, psyllids, whiteflies and thrips [17–20].

Among the vector borne viral diseases of citrus, citrus tristeza (CTV) which is caused by a *Closterovirus* is the most dominant and widely studied viral diseases of citrus. It is transmitted by different aphid species primarily by black citrus aphid (*Toxoptera citricida* Kirk.) and cotton-melon aphid (*Aphis gossypii* Glov.) [17, 21]. Another emerging viral disease of citrus is the yellow vein clearing (CYVCV) caused by a *Mandarivirus*. It was first observed in Pakistan in 1988 in the orchards of sour orange (*C. aurantium* L.) and lemon (*C. limon* L.) [22], and later on it was reported in China, India, Iran and Turkey [23–26]. This CYVCV is reported to be vectored by e transmitted by whiteflies and aphids (*Aphis craccivora* and *A. spiraecola*) [25, 27]. Although not virus borne, citrus stubborn is a destructive disease being caused by a bacterium *Spiroplasma citri*. It is usually transmitted by many species of leafhoppers, primarily by *Scaphytopius nitridus* and Circulifer tenellus in citrus-growing suburbs of California and Arizona and by *Circulifer haematoceps* in the Mediterranean zones [17].

4. Indexing strategies

Indexing is an indispensable procedure to produce and diagnose disease-free plants. Different techniques or combination of techniques have been applied in this regard and the effectiveness of each depends upon the facilities available. Generally indexing can be divided into two types.

A. Field indexing; also known as biological indexing including the mechanical inoculation through direct contact or vegetative propagation and/or through insect transmission.

B. Laboratory indexing; also known as quick indexing including serological, molecular and chemical assays.

Commonly used indexing methods are tissue grafting, budding, insect transmission for biological indexing and enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) for quick indexing strategies. Although all viruses and virus-like pathogens can be detected through PCR and its derivatives, polyacrylamide gel electrophoresis (PAGE) is commonly used for the detection of viroids.

4.1 Biological indexing

Biological indexing is the inoculation or introduction of virus source (infected sample) into the indicator plants for detection and purification. It involves one of the common indexing methods such as vegetative propagation of infected scion (grafting/budding) to indicator plants, mechanical inoculation of indicator plants or transmission of virus through the insect vector (*e.g.* aphids for CTV, psyllids for greening and leaf hoppers for prokaryotic diseases). Biological indexing is usually

Sr. No.	Disease	Appropriate number of test plants	Indicator plants (for inoculation)	Favorable temperature	Symptoms on the indicator (indexed) plant
1.	Citrus tristeza	5	Mexican lime (C. aurantifolia), sweet orange on sour orange root stock, Duncan grapefruit	65–68° F Cool-warm	Vein clearing, stem pitting, leaf cupping, decline on sour orange stem pitting on grapefruit.
2.	Yellow vein clearing	5	Lemon (C. limon), sour orange (*C. aurantium*)	Cool	Yellowing and clearing of veins
3.	Ringspot/ Infections variegation	4	Citron (C. medica), cowpea (Vigna unguiculata)	Cool	Ringspot, necrotic local lesions, distorted leaves.
4.	Psorosis	4	Sweet orange (C. sinensis)	Cool	Flecking on leaves
5.	Cachexia-xyloporosis	5	Mandarin (C. reticulata) Parsons special	Warm >95°F	Gum in bark, scion and at bud union
6.	Exocortis	5	Citron (*C. medica*)	Warm	Tip browning, leaf epinasty
7.	Cristacortis	4	Grapefruit, sweet orange	Cool- warm	Flecking
8.	Concave gum	4	Sweet orange	Cool	Oak leaf pattern, narrowing of leaves
9.	Citrus greening disease	5	Mandarin (*C. reticulata*), Grapefruit (*C. paradisi*), Murraya sp.	Cool- warm	Leaf blotches, chlorosis
10.	Citrus stubborn disease	4	Sweet orange, grapefruit, periwinkle (Catharanthus roseus)	Warm	Stunted shoot, smelling of leaves, Zn deficiency like signs.
11.	Yellows (probably Aster)	5	Grapefruit, Periwinkle (C. roseus)	Warm	Chlorosis
12.	Bud union crease	5	Sweet orange	Warm	Brown line at bud union
13.	Gummy bark	5	Mandarin	Warm	Gum in the bark

Note: *The above information is extracted from the work of [10, 12] and personal communication of Dr. S.M. Mughal. (Dr. Mughal was also in the team of Dr. Catara during the surveys of citrus growing areas of Pakistan in early 1980's).*

Table 3.
Biological indexing of citrus graft-transmissible pathogens.

time consuming, require glasshouse facilities and takes about 6–12 months for results. At least 3–4 plants are required per treatment. Biological indexing of graft-transmissible pathogens, indicator plants and symptoms in the indicator plants are summarized in **Table 3**.

Detailed methodology for biological indexing has been described much in literature [28–31]. Followings are the generalized and simplified steps to be kept in mind during the biological indexing on the basis of available literature.

i. Sow the seeds of test plants (usually Mexican lime or acid lime) in the sand in germinating tray. Transplant the seedlings in pots having potting media

(sand, soil and moss @ 1:1:1 ratio) after 17–28 days of germination, depending on the germinating conditions.

ii. Inoculate the seedlings at 4–6 leaf stage.

iii. Keep the indicator plants in insect-free chambers before and after inoculation.

iv. **Mechanical inoculation:** grind the virus infected samples in phosphate buffer (pH 7.2). Dust the carborundum powder on to the indicator plants, but not too much, before inoculation (excessive dusting of carborundum will cause the necrosis and misled results).

v. Pass the crude virus extract from double layer muslin cloth and then apply to the indicator plants with the help of forefinger and leave for 3–4 min. Remove the excess sap from indicator plants under tap water.

vi. **Insect transmission:** Do rearing of vector insects in the laboratory or collect the vector insects directly from the field and keep them in laboratory for few days to be acclimatized with the rearing conditions. Provide them with the artificial or natural diet.

vii. Observe the fasting period according to the nature of transmission (non-persistent, persistent or semi-persistent) before allowing them to be fed on virus source to acquire the virus.

viii. For non-persistent transmission pre-acquisition fasting time is 30–90 min. Long fasting period enhances the chances of quick acquisition of virus from the infected source.

ix. Transfer the insects on to the young leaves with virus symptoms/virus infected plants with the help of camel brush and allow them to feed for few min.

x. After few min, immediately transfer the viruliferous insects on to the indicator plants and keep the plants in insect-free chamber to avoid the contamination from other insects.

xi. Maintain the insect population on indicator plants for at least 24 hr. and eradicate them after that through insecticides.

xii. For persistent transmission and semi-persistent transmission pre-acquisition fast has no effect. In both cases long acquisition feeding period enhances the chance of transmission.

xiii. Maintain the insect population for a week and eradicate them with insecticide in case of semi-persistent transmission while maintain the insect population through transferring them on new indicator plants till they are alive.

xiv. **Vegetative propagation:** Take the infected bud-wood from the virus source and graft on to the rootstock/indicator plants and keep the indicator plants in insect-free zones.

xv. Temperature range between 65 and 95°F helps the appearance of symptoms on indicator plants for viruses and virus-like pathogens. Observation time also varies from 3 to 16 months for different viruses, virus-like pathogens and viroids.

Laboratory indexing/advanced detection methods

There are rapid methods, highly specific, routinely applicable and some of which test large number of samples. These methods are summarized in **Table 4**. ELISA is the main laboratory indexing method used for the detection of CTV, PAGE for viroids and PCR for all diseases. Mother plants (plants recovered by nucellar embroyony *in-vivo* or *in-vitro*, by thermotherapy or micro-grafted plants or by micro-budding may be indexed by any of the above methods. Although 'chromatography' is a useful in chemical indexing of certain virus and virus-like pathogens but it is less reliable than vegetative propagation indexing. Electron microscopy is also helpful for the detection of greening and stubborn diseases other than the viruses. Moreover, *S. citri* can be cultured on a specific medium. Now-a-days, commercial kits are available for the ELISA, PCR and other detection methods along with the instructions.

4.2 Serological assays

Serology involves the quick indexing of plant viruses, based on the antibody–antigen reaction. Enzyme-linked immunosorbent assay (ELISA) is one of the widely

Sr. No.	Methods	Tested for	Advantages and limitations	References
1.	Immunofluorescence, tissue staining Azure A, Light microscopic observations	Citrus tristeza virus, yellow vein virus, greening diseases	Simple, economical, limited number of samples, time consuming, non-specific	[29, 32]
2.	Gel immunodiffusion	Citrus tristeza virus	Economical, time consuming, require quality antiserum, where ELISA facilities are not available.	[6]
3.	Enzyme-linked Immunosorbant Assay (ELISA and its variants)	Citrus tristeza virus and some prokaryotes	Rapid, economical, specific, routinely applied for large number of samples, quantitative, sensitive	[33]
4.	Electron microscopy (EM)	Citrus tristeza viru and other viruses	Quick for elongated viruses (CTV, CYVV), requires proper facilities	[6]
5.	Immunosorbant electron microscopy (ISEM)-Decoraton Technique	Citrus tristeza virus	Quick, specific, require antiserum and proper facilities, limited sampling	[6]
6.	Polyacrylamide Gel Electrophoresis (PAGE)	Citrus viroids (Exocortis, Cachexia)	Excellent for viroid detection and characterization, requires purification of viroids and proper conditions and facilities	[34]
7.	Molecular hybridization (RNA/DNA Probes), Polymerase Chain Reaction (PCR)	Virus and virus like diseases, CTV.	Highly sensitive, routinely applicable, time consuming, require primers and equipment facilities	[6]

Note: *Large scale screening of material is possible with any of the method(s) mentioned above. However, there are several limitations including time and availability of proper facilities and trained manpower.*

Table 4.
Advanced methods for the detection of viral diseases of citrus.

used in detection of plant viruses. It is relatively cheap and can test large number of samples.

ELISA with its derivatives, direct (DAS-ELISA) and indirect (DAC-ELISA), is the main serological indexing tool used for most of the citrus viruses at large scale samples.

Followings are some general steps followed during the ELISA based detection or indexing [35].

i. **For DAS-ELISA;** Coat the antibodies in the ELISA plate and keep at 4°C for overnight or 37°C for 4 hr. for incubation.

ii. Wash the plate with washing buffer for 3 times with the interval of 5 min.

iii. Add the antigen (virus sap extracted from infected samples) into the wells of ELISA plate and incubate as above.

iv. Repeat the washing and coat the ELISA plate with enzyme-labeled antibodies and incubate as above.

v. Repeat the washing step and add the substrate followed by incubation for 30 to 90 min for visual observation of color change and read the micro-plate through ELISA reader/spectrophotometer for quantitative data.

vi. **For DAC-ELISA:** Add the antigen and incubate the plate as above.

vii. Wash the ELISA plate as in DAS-ELISA.

viii. Add the primary antibody and incubate.

ix. Add the secondary antibody and incubate.

x. Add the enzyme-labeled antibodies and incubate.

xi. Add the substrate and then observe the color change after incubation and read the plate through ELISA reader for quantitative data.

Note: Repeat the washing step after every step before adding the substrate. Stop the reaction in both types of ELISA with the help of 1 N NaOH.

4.3 Molecular assays

Molecular detection of citrus viruses and virus-like diseases has revolutionized the subject and provided the platform to detect the early stages of infection to reduce the economic losses. The molecular hybridization techniques supplemented with nucleic acid amplification methods based on PCR, in which high-throughput sequencing approaches can be adopted to identify the strains in relation to evolutionary history or phylogenetic assemblages [36, 37]. Although, nucleic acid based methods are highly sensitive and discriminatory allowing specific strain typing, but it bears the problems in reproducibility [38, 39]. Progressive efforts have been made to decrease the troubleshoots and hurdles to improve the amplification systems by improving the sensitivity and specificity of detection by limiting the high contents of plant related enzyme inhibitors. In contest, nested and multiplex PCR

provides high sensitivity and make the possible to detect several targets in single assay [40]. Moreover, highly sensitive technologies by conducting the amplification of nucleic acids in an isothermal reaction, nucleic acid sequence-based amplification (NASBA) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) provides specific detection of viruses and virus-like diseases.

The addition of real-time PCR for high-throughput testing allows the automation of PCR by combing the fluorimeteric approaches to detect and quantify the targets simultaneously [41, 42]. The combination of different protocols including the serological techniques and molecular approaches will increase the accuracy and reliability of virus diagnostic. Furthermore, in future prospects, nucleic acid arrays and biosensors assisted by nanotechnology will open new corridors to revolutionize the detection of plant viruses and virus-like diseases.

Citrus tristeza virus (CTV) is the most dangerous citrus disease all over the world and is also known as quick decline disease reducing the population of citrus trees significantly [43–45]. However, the utilization of advanced diagnostic methods, such as, biological indexing, electron microscopy (EM), ELISA and PCR or reverse transcriptase PCR (RT-PCR) is providing promising detection of the virus particles and leading towards the management strategies of CTV [46]. The application of conventional PCR is sensitive and specific under optimized and controlled conditions. However, sometimes, it is not possible to judge the amount of pathogens in the samples. Therefore, researchers have to employ other subsequent techniques for complete detection and quantification. Meanwhile, with real-time PCR approach, users can monitor the reaction and also the quantification of the specific pathogen in the sample. While setting up the real-time reaction for virus detection, it is the basic requirement to adapt the specific conditions of the detection system and instrument, and the characteristics of the reaction reagents and cycling procedures in which the most important are primer design, reaction components and conditions. The real-time PCR works well with small amplicons (5–200 bp), while standard PCR allows amplification of several hundred bases without sensitivity and specificity. Moreover, concentrations of $MgCl_2$, primers, and dNTPs are usually higher than conventional PCR [47].

The new developing chemistries are setting up the protocols with different characteristics depending upon the target and assay requirements. In addition to the most widely working chemistries (SYBRGreen, TaqMan, Scorpion, Molecular Beacons), there are more novel chemicals or technologies such as Amplifluor; Locked Nucleic Acid (LNA) Probes, Sigma Proligo; Cycling Probe Technology (CPT), Takara; Light Upon eXtension (Lux) Fluorogenic Primers, Invitrogen Corporation; Plexor Technology, Promega [48, 49]. Real-time technology is being used also in multiplex formatting for the specific detection and strain identification for several viruses [50–55]. Furthermore, real-time reaction in multiplex system is difficult to optimize due to different ratio between the targets and the reaction. The replacement of conventional PCR with real-time PCR is providing new horizons towards the multiple detection system of plant viruses especially of the citrus viruses and virus-like diseases.

5. Detection of citrus viroids

After the discovery of viroid group of pathogens as an infectious agent to the plants, new aspects in virology were come in front of researchers to be addressed. Viroids are the smallest pathogens which consist of 246 to 401 nucleotides. They are

low molecular weight, circular and single stranded RNAs. Viroids exist as free RNA because they lack protein coat [56]. Since viroids do not code for protein and enzyme, they rely on host enzyme for protein synthesis system and replication. To date, 38 viroids have been identified and they are classified into 2 families *i.e. Pospiviroidae* and *Avsunviroidae* [57].

The major economic important viroids in different plants are coconut viroids (CCCVd), citrus viroids (Exocortis and cachexia and variants), Hop stunt viroid and Potato spindle tuber viroids [57]. The origin of viroids is still questionable as they do not have natural host relationship [58, 59].

Citrus production is also affected by viroids. These are the emerging threat to citrus industry. To date, seven citrus viroids have been detected so far in citrus *viz*. *Citrus Exocortis viroid* (CEVd), *Citrus Bent Leaf viroid* (CBLVd), *Hop Stunt viroid-citrus* (HPSVd-cit), *Citrus Dwarfing viroid* (CDVd), *Citrus Bark Cracking viroid* (CBCVd), *Citrus viroid V* (CVd V) and *Citrus viroid VI* (CVd VI-OS). These have been distributed in different geographical areas as shown in **Table 5** [70]. Diseases caused by citrus viroids are citrus exocortis disease (CED), citrus cachexia disease (CCD), citrus leaf bending disease (CLBD), citrus bark cracking disease (CBCD) and citrus dwarfing disease (CDD). Among these, citrus exocortis and citrus cachexia-xyloporosis are the most devastating and widely distributed [57]. These diseases cause a reduction in yield, size of fruit and quality of production [8]. These are transmitted directly and through propagation [71]. Stunting, dwarfing, bark cracking, yellowing of leaves, backward leaf bent, pin holing, yield loss and ultimately tree decline are the common symptoms of citrus viroid diseases [63, 71, 72]. Citrus viroids alone or with other viruses or prokaryotes in the host contribute considerably in tree decline [73]. Exocortis and cachexia are the major viroids which are widely distributed in citrus orchards. Other citrus viroids have also been detected from citrus orchards in different parts of the world [73]. Unlike viruses, viroids do not have protein coat, therefore, these are very difficult to detect through serological methods. For this purpose, molecular techniques such as PCR, PAGE are available for the detection of citrus viroids. These are sensitive, sophisticated and rapid detection techniques. Molecular techniques not only help in the detection but also in the characterization of viroids.

Viroid	Geographical distribution	References
Citrus excocortis viroid (CEVd)	Australia, Argentina, Brazil, Japan, Taiwan, Corsica, China, India, Israel, Spain, Pakistan, South Africa USA, Uruguay, Iran	[8, 57, 60–62]
Citrus bent leaf viroid (CBLVd)	Israel, Japan, Australia, China, Uruguay, Pakistan, UAE, Iran, Spain	[8, 60, 61, 63–65]
Hopstunt viroid (CVd- II),	Israel, Brazil, Uruguay	[8, 60, 62]
Citrus dwarfing viroid (CDVd)	USA, Uruguay, Pakistan	[8, 60, 65, 66]
Citrus bark cracking viroid (CBCVd)	USA, Uruguay	[60, 67]
Citrus viroid V (CVd-V)	Spain, Iran	[61, 68]
Citrus viroid VI (CVd- VI)	Japan	[69]

Table 5.
Geographical distribution of citrus viroids.

Genus	Citrus viroid	Length (nucleotides)	Diseases
Pospiviroid	*Citrus excocortis viroid* (CEVd)	371	Citrus exocortis disease
Hostuviroid	*Hop stunt viroid-citrus* (CVd-IIa) *Citrus cachexia viroid* (CVd-IIb)	299–302	Citrus cachexia disease.
Cocadviroid	*Citrus viroid IV*	284–286	Citrus bark cracking disease
Apscaviroid	*Citrus bent leaf viroid* (CVd-I),	318	Citrus leaf bending disease
	Citrus dwarfing viroid (CVd-III)	294–297	Citrus dwarfing disease
	Citrus viroid V	295	
	Citrus viroid VI	330	

Note: *The above information is extracted from the work of Hammond & Owens (2006) and King et al. (2011).*

Table 6.
Classification of citrus viroids (king et al., 2011; Hammond & Owens, 2006).

5.1 Pathogen description and characterization

All citrus viroids are classified in different genus under *Pospiviroidae* as mentioned in **Table 6**.

5.2 Diagnostic methods for citrus viroids

Biological indexing is done through graft inoculation in indicator plants. It is very suitable to check the symptoms produced by citrus viroids and their severity. The most important host for indexing CEVd is Etrog citron (*Citrus medica*, Arizona 861) because of its great sensibility and rapid symptom expression [74]. According to Nakahara et al. [75], bioassay on Etrog citron is the most sensitive technique in detection of viroids although it takes more time compared to other methods. **Molecular tools** are now widely being used in the detection of citrus viroids. Combinations of several molecular techniques are very useful for reducing the time and to allow large numbers of samples to be examined and to identify each citrus viroid species [75, 76]. **PAGE** is also used to separate variation based on molecular weight. PAGE is not suitable for indexing large number of samples because it is not cost-effective. It is used to test the circularity of viroid RNA by two-dimensional denaturing PAGE (2D-PAGE) [77–79]. Sequential-PAGE is also commonly used and capable of detecting all citrus viroids [71].

Reverse transcription- polymerase chain reaction (RT-PCR) is the most commonly used method to detect citrus viroids. It is also a reliable method for quick screening and detection of citrus viroids [80]. It is known for its high specificity and ability to detect unknown viroids or variants [57]. **Multiplex RT-PCR** is another approach to detect simultaneously more than a viroid by using several set of primers. For instance, CEVd, CBLVd, CVd 1-LSS, CVd-II, CVd-III, CVd-IV and CVd-VI were successfully detected simultaneously via multiplex RT-PCR [69]. **Real-time RT-PCR** is also used to detect citrus viroid. It is a quantitative PCR technology basically same as RT-PCR but it measures and quantifies products generated during each cycle of PCR [81]. **Molecular hybridization** is based on the specific interaction between complementary purine and pyrimidine bases forming A-U and G-C base pairs. According to Targon et al. [82], imprint hybridization technique is fast, sensitive and economic methods to be used as a routine for citrus viroid indexing in the certification programs. However, dot-blot technique is required an appropriate amount of extracted nucleic acids [75], and it is not suitable

for detection of new or unknown viroids. Another molecular approach to detect viroids is Northern blot hybridization. CEVd, CBLVd, CVd-II, CVd-III and CVd-VI were successfully detected by Northern blot hybridization using specific probes in inoculated Etrog citron [83].

5.2.1 RT-PCR for detection of citrus viroids

5.2.1.1 Samples collection

Collect the leave samples based on virus and viroids-like symptoms in the field. Bring the leaves samples to laboratory for processing and preservation until use as follows;

a. Collect the leave samples in sterile plastic bags and place in ice box.

b. In the laboratory, wash the samples first in 10% bleach followed by distilled water.

c. Dry the samples and put in the plastic bags.

d. Label the plastic bags and store them at −80°C until further use.

5.2.1.2 Nucleic acid extraction

Extract the nucleic acids from leave samples using the TESLP buffer [84] as follows;

1. Grind the 2-3 g of leaves (about 10–12 leaves) using mortar and pestle with liquid nitrogen. The slurry needs to be transferred to 50 ml screw cap tubes.

2. Add 10 ml of TESLP buffer [0.13 M Tris–HCl (pH 8.9), 0.017 M EDTA (pH 7.0), 1 M LiCl, 0.83%SDS, 5%PVP] into the tube.

3. Add 16 μl of 2-mercapthoethanol into the mixture.

4. Incubate the mixture for 30 min at room temperature in the rotary shaker.

5. Centrifuge the mixture at 11000 rpm for 15 min.

6. The supernatant needs to be transferred to a new 50 ml screw tube.

7. Add phenol:chloroform:iso-amyl (PCA, 25:24:1) @ 3:2 and mix well using vortex followed by centrifugation for 15 min, 11,000 rpm at room temperature.

8. Transfer the supernatant into a new 15 ml screw tube and add CA (24:1) @ 4:3. The mixture needs to be mixed well using vortex and repeat the step 7.

9. The supernatant is obtained into a new 15 ml screw tube @ 1 volume of supernatant with 0.9 volumes of 90% isopropanol.

10. The tube is inverted 3–4 times to mix the components. Do not vortex or centrifuge.

11. The mixture is incubated at −80°C for 30–40 min (or -20°C for 3–4 hr. or overnight).

12. The mixture is centrifuged for 15 min, 11,000 rpm at room temperature.

13. The isopropanol is discarded and the pellet obtained is transferred into 1.5 ml micro centrifuge tube.

14. The pellet is washed with 1 ml of 70% ethanol followed by washing with 1 ml absolute ethanol until the clean pallet is obtained.

15. The pellet is suspended in 50 µl of sterile double distilled water.

16. The pellet is immediately used for RT-PCR or stored in -20°C until use.

Reverse Transcription Polymerase Chain Reaction [69]:

5.2.1.3 Synthesis of cDNA

The extracted RNA is used to run RT-PCR. Reverse Transcription process is carried out in two steps to synthesis cDNA as follows;

Step 1: (1X)

Experimental RNA = 5 µl
Reverse primer = 1 µl
Double distilled water = 2.5 µl
Total Volume = 8.5 µl
The reaction is incubated at 80°C for 12 min then immediately transferred to ice for 5 min.

Step 2: (1X)

AMV-RT = 1 µl
dNTPs = 2 µl
RNAse Inhibitor = 0.5 µl
$MgCL_2$ = 4 µl
RT buffer = 4 µl
Total volume = 11.5 µl

The reaction is incubated at 55°C for 30 min. After 30 min, the process is stopped when it reaches to 10°C. The cDNA obtained is stored in −80°C freezer until use (or it can be used immediately).

5.2.1.4 PCR protocol

The final volume of PCR should be 25 µl which consists of 12.5 µl of PCR master mix, 5 µl of cDNA, 5.5 µl of sterile double distilled water, 1 µl of forward primer and 1 µl of reverse primer.

The conditions for PCR amplification (35 cycles) are as follows:

a. Denaturation:

1. 94°C for 10 min

2. 94°C for 30 seconds

3. 60°C for 1 min

b. Annealing at 60°C for 10 seconds.

c. Extension at 72°C for 10 seconds and then 5 min.

The list of specific primers used is given in **Table 7**.

5.2.1.5 Agarose gel electrophoresis

The amplified RT-PCR product is separated using 2% agarose gel as follows [85];

- 2% agarose gel is prepared with 1x TBE buffer
- Samples are loaded in the gel and electricity is provided at 100 volts for 50 min.
- The gel is stained with Ethidium bromide for 10 min and washed with distilled water for 5 min.
- The gel is visualized under Trans UV and captured with Gel Doc XR system.

5.2.1.6 PCR product purification

Positive PCR products with expected size are purified using MinElute® Gel Extraction Kit according to the standard protocol provided with Kit.

1. The expected size of band is excised from the agarose gel with a sterile, sharp scalpel.

Viroid	Type	Sequence	Target (Product size)
CEVd	RT-Reverse	*5′ -CCGGGGATCCCTGAAGGACTT-3'*	371 bp
	PCR-Forward	*5′ -GGAAACCTGGAGGAAGTCGAG-3'*	
CVd-I	RT-Reverse	*5′ -TCGACGACGACCAGTCAGCT-3'*	233 bp
	PCR-Forward	*5′ -TCCCCTTCACCCGAGCGCTGC-3'*	
CVd-I-LSS	RT-Reverse	*5′ -ACGACCGCTCAGTCTCCTCT-3'*	247 bp
	PCR-Forward	*5′ -CTGTAACCGGACCGGTCTCCTTC-3'*	
CVd-II	RT-Reverse	*5′ -CCGGGGCTCCTTTCTCAGGTAAGT-3'*	302 bp
	PCR-Forward	*5′ -GGCAACTCTTCTCAGAATCCAGC-3'*	
CVd-III	RT-Reverse	*5'-TCACCAACTTAGCTGCCTTCGTC-3'*	271 bp
	PCR-Forward	*5′ -CTCCGCTAGTCGGAAAGACTCCGC-3'*	
CVd-IV	RT-Reverse	*5′ -TCTATCTCAGGTCGCGAAGGAAGAAGC-3'*	209 bp
	PCR-Forward	*5′ -TCTGGGGAATTTCTCTGCGGGACC-3'*	
CVd-VI	RT-Reverse	*5′ -GTCCGCTCGACTAGCGGCAGAGAGC-3'*	166 bp
	PCR-Forward	*5′ -CGTCGACGAAGGCATGTGAGCTT-3'*	

Table 7.
List of specific primer for citrus viroids [69].

2. The gel slice is put in a sterile 1.5 ml micro-centrifuge tube and weighed.

3. QC buffer, provided with the kit, is added @ 3:1 volume of gel.

4. The gel slice is incubated at 50°C for 10 min until the gel slice has completely dissolved.

5. The mixture is vortexed every 2–3 min to facilitate the dissolution of gel slices.

6. Then, 1 gel volume of isopropanol is added and mixed by inverting with pipette.

7. The MinElute spin column is placed into 2 ml collection tube.

8. The sample is transferred into the MinElute column and centrifuged at 13000 rpm for 1 min.

9. The flow-through is discarded and put back the column into the same collection tube. 750 µl of Buffer PE is added to MinElute column and let it stand for 1–2 min.

10. Centrifuged at 13000 rpm for 1 min and the flow-through is discarded.

11. The process is repeated to remove Buffer PE completely.

12. The ethanol residual left at the bottom of the column is discarded and MinElute column is placed into a sterile 1.5 ml micro-centrifuge tube.

13. 30 µl of EB Buffer is added to the center of MinElute membrane to elute DNA. The mixture is let to stand for 1 min, and then is centrifuged at 13000 rpm for 1 min.

14. MinElute column is discarded and the tube is stored in - 20°C.

5.2.1.7 Molecular Cloning (TOPO TA cloning kit, Invitrogen)

Positive PCR samples will be cloned using the TOPO TA cloning kit according to the standard protocol provided along with the Kit as follows;

Ligation

- 4 µl purified PCR products.
- 1 µl vector (pCR2.1-TOPO).
- 1 µl Salt solution.
- Incubate in PCR machine/ heat block at 25°C for 30 min.
- Add 2 µl of ligation mixture into competent cell *E. coli* - do not pipette up and down, just thaw a bit/ swirl.
- Put 30 min in ice.

Transformation

- Put 30 sec in 42°C water bath (heat shock).
- Put in ice for 5 min (immediately after heat shock).
- Add 250 μl SOC medium to mixture-seal competent cell tube with parafilm.
- Put at 200 rpm in 37°C incubator shaker for 1 h 30 min.
- Warm the petri dish in incubator for 20–30 min.
- Spread 40 μl X-gal on petri dish (LBA media).
- After spread the X-gal, put the petri dish in incubator for 20–30 min.
- Finally, spread the sample mix on petri dish and incubate overnight at 37°C.

Note: Strictly follow the incubation time and temperature in the protocol during cloning.

5.2.1.8 Two Dimensional poly acrylamide gel electrophoresis (2D PAGE)

2D PAGE is carried out to for the detection and to check the circularity of Viroid RNA. Following is the recipe and protocol for PAGE.

Gel Ingredients

- Acrylamide (A)
- Bisacrylamide (B)
- 40% AB in 50 ml distilled water @ 19:1 ratio

Non Denaturing Gel:

Ingredients	8% GEL	5% GEL
40% AB	6 ml	6.25 ml
10X TBE	3 ml	5 ml
dH_2O	20.25 ml	37.7 8 ml
10% APS	750 μl	937.5 μl
TEMED	40 μl	43.75 μl
Total Volume	30 ml	50 ml

- Mix the gel with magnetic bar.
- Wash the glass with KOH and dH_2O.
- KOH washing buffer includes 10 g KOH + 10 ml dH_2O + 90 ml and 99% ETOH.
- Rinse the glass with dH_2O and let it dry.

- Prepare the gel and cast into electrophoresis set.
- Let the gel to polymerase for 30 min.
- Pre-run empty gel for 20 min at 10 mA.
- Pre-run sample for 10 min at 10 mA and then run sample for 1 hr. plus at 20 mA.

5% Non-denaturing Gel

- 40% AB: 8.7 ml
- Urea: 25.2 g
- 10XTBE: 5.25 ml
- 10% APS: 750 µl
- TEMED: 45 µl
- dH2O: 17.25 ml
- Total volume: 52.5 ml

Fixer 1:	
ETOH (10%, V/V)	10 ml
Acetic Acid (5%, V/V)	5 ml
dH_2O	85 ml
Fixer 2:	
ETOH (10%, V/V)	10 ml
Acetic Acid (5%, V/V)	0.5 ml
dH_2O	89.5 ml
Silver Solution:	
Silver Nitrate	0.3 g
dH_2O	150 ml
Developer Solution:	
3 mM $NaBH_4$	0.023 g
Formaldehyde	0.75 ml
0.375 M NaOH	3 g
dH_2O	200 ml

Silver Staining:

- Fix the gel in fixer 1 for 10 min at room temperature in shaker.
- Fix the gel in fixer 2 for 10 min at room temperature in shaker.

- Dip the gel in silver stain solution for 1 hr. under dark condition.
- Rinse with dH_2O twice with the interval of 1 min.
- Add developer solution in the end.
- Stop developing by adding 5% acetic acid.

5.3 Citrus tristeza virus (Ctv): a case study

5.3.1 Introduction

CTV belongs to the genus *Closterovirus* of the family *Clostervoiridae*. Virus particle is a monopartite, positive sense, comprising of ssRNA genome of approximately 20Kb in size. It is the largest known form of a plant virus and its genome is encapsulated in a flexuous rod 2000 nm long particles composed of coat protein subunits of 25KDA [86–89]. ssRNA genome comprised of 19,296 nucleotides that encode for 12 open reading frames [90]. CTV probably originated in Asia and has been spread to all citrus growing areas by infected plant material movement and now is widely distributed to all major citrus growing areas as summarized in **Table 8**. Over the two decades *i.e.* 1930–1950, millions of citrus trees were destroyed due to CTV infection and citrus orchards were almost wiped out in Brazil, Spain, and Argentina. This virus was the killer of three million citrus trees grafted on sour orange rootstock alone in south California [91–94]. The tristeza disease was first reported in Florida in 1959 and by 1980s became the serious threat to citrus industry [95]. By 1991, an estimation of total world loss of 100 million trees was recorded due to CTV in Argentina, Brazil, Spain, California, Venezuela and other areas [96, 97]. Several strains of CTV have been identified primarily on the basis of their biological reaction in several citrus species and indicator plant. The major groups of strains are mild that cause barely detectable clearing of leaf veins in Mexican lime; decline–inducing strains cause death of trees when propagated on sour orange rootstock. Stem pitting strains cause mild to severe pitting of stems and branches of grapefruit and orange resulting in low yield [95, 98]. Almost all the citrus varieties and hybrids have been infected with CTV [91]. Symptom expression of CTV in citrus hosts is highly variable and depends upon host species (rootstock and scion combination), virulence of CTV isolates and soil or environmental conditions. Characteristics symptoms of CTV are vein clearing, decline, stem pitting, seedling yellows, stunting and leaf corking on different citrus hosts like sweet orange, grapefruit, grafted on sour range root stock. Severity of infection and symptoms expression on cultivars vary from mild to severe isolates [99–101]. CTV is transmitted in nature by different species of aphids in a semi-persistent manner and through grafting [102, 103]. The most efficient vector involved in semi-persistent manner is *T. citricida* Kirkaldy (brown or black citrus aphid) when compared with other aphids.

5.3.2 Indexing

Serological and biological indexing: Indexing includes biological, serological and molecular methods, which are the common procedures according to their reliability, sensitivity and duration to detect the CTV. During a survey in Spain, 22 CTV isolates were collected on the basis of geographical information, source tree and symptomology and then were characterized by biological indexing. Diversified

Regions	Countries	Status
EPPO	Israel, Spain, Turkey	Present
	France	Found but not established
	Algeria, Cyprus, Egypt, Italy, Morocco, Tunisia	Scattered infection
Asia	Brunei, China, Georgia	Present
	India	Widespread
	Indonesia. Iran, Japan	Present
	Jordan	Unconfirmed
	Korea, Malaysia, Nepal	Present
	Pakistan	Present (Scarce Information available)
	Philippines, KSA, Sri Lanka, Taiwan, Thailand, Viet Nam, Yemen	Present
Africa	Cameroon, Chad, Ethopia, Gabon, Ghana, Kenya, Mauritious, Mozambique, Nigeria, South Africa, Tanzania, Zaire, Zambia, Zimbabwe	Present
	Libya	Unconfirmed
North America	Bermuda, Mexico, USA	Present
Central America and Caribbean	Antigua, Barbuda, Bahamas, Belize, Costa Rica, El Salvador, Guatemala, Honduras, Jamaica, Netherlands Antilles, Nicaragua, Puerto Rico, St. Lucia, Trinidad, Tobago	Present
	Dominica	Unconfirmed
South America	Argentina, Bolivia, Brazil	Present (wide spread)
	Chile	Found, not established
	Colombia, Ecuador, Guyana, Peru, Paraguay, Suriname, Uruguay	Present
Oceania	American Samoa, Australia, Fiji, New Zealand	Present

Source: Anonymous, 2004.

Table 8.
Geographical Distribution of Citrus tristeza closterovirus.

symptoms were produced on 9 indicator species. Mexican lime was found to be a good indicator host [104].

In Morocco, 14 diverse isolates were selected from samples during survey and then characterized on the basis of reaction pattern. Among these 14 isolates, four were severe and two were mild isolates. Isolates were also indexed against a series of monoclonal antibodies [105]. DAS-ELISA was used to detect the CTV from the samples collected during a survey in Western and Midwestern development regions of Nepal [106]. One hundred and eighty-eight samples were analyzed through biological indexing and DAS- ELISA to detect tristeza, psorosis and similar diseases like-symptoms including viroids in orange varieties in all the regions and the cachexia was detected as the most important and widespread disease [107]. Biological indexing is still considered as an important tool using for the characterization of CTV isolates. Different strains were identified through symptoms expression on differential hosts, including Mexican lime and sweet orange. Moreover, they

observed visual symptoms of different strains on Mexican lime and sweet orange through biological indexing followed by ELISA [108]. Detection of CTV in Spain was compared by indexing using monoclonal and polyclonal antibodies [109].

Molecular indexing: Different nucleic acid based indexing methods have been developed for the quick detection of CTV. The adaptability of these methods depends upon the reliability, time duration and sensitivity. Alteration in protein patterns in rootstock bark from CTV infected tree were analyzed through PAGE [110]. There was a clear modification in protein pattern but not in CTV free trees. Similarly, Northern blot technique was used to compare dsDNAs extracted from CTV infected and CTV free plants. Two out of the three CTV isolates were detected by this method [111]. CTV was also detected in the three aphid species through RT-PCR. IC-RT-PCR was used to amplify the coat protein gene [112]. Sensitivity of cDNA probe was slightly better than hybridization with ^{32}P-labeled probe. Similarly, hybridization with tissue print with DIG-probe could differentiate CTV isolates grown under green house or field conditions [113]. In Taiwan, RT-PCR was found to be a rapid and sensitive assay than other serological methods but one step RT-PCR, which is the combination of reverse transcriptase and polymerase chain reaction in one tube. It is more sensitive and detects the CTV when virus concentration is very low. Comparison between ELISA and RT-PCR revealed that ELISA was better than RT-PCR at detecting mild CTV strains as the virus was detected in all parishes, while RT-PCR detected CTV in only 8 parishes. It would appear that the primers used for RT-PCR are more specific for severe CTV isolates [114]. Some modifications were introduced in PCR-ELISA to increase its sensitivity and reduced the costs of detection. PCR-ELISA is the immune-detection of PCR products and effective for detection and differentiation of plant viral nucleic acids. PCR-ELISA being a laborious and expensive method was modified and simplified by using asymmetric PCR. It made PCR-ELISA more sensitive than TaqMAN™, a fluorescence-based detection method.

Three microscopy procedures for detecting CTV were compared which provided additional alternatives for very rapid CTV indexing, including the use of EM, SSEM and light microscopy. In light microscopy, inclusions were found in young phloem tissues of all CTV-infected hosts examined. Similarly, in SSEM virus particles were found on grids prepared with antiserum and extracts from infected tissue. CTV particles could be detected in pooled samples representing one in 100. Similarly, virus particle fragments were observed infrequently in samples representing one infected plant in 1,000 samples [32].

6. Conclusion

Citrus is an important fruit crop of the world and has a great potential for local consumption, export purposes and industrial uses. Unfortunately, citrus orchards are facing the problem of low productivity due to citrus decline. This is mainly attributed, among other factors to the prevalence of graft-transmissible virus and virus-like diseases, unhygienic nursery operations and poor orchard management. However, most of the problems arise from nurseries. It is the time that the nurseries should operate on highly technical and scientific lines and should work on providing disease-free and certified plants to the citrus growers. To establish the disease free nurseries, indexing of virus and virus-like diseases are the major area that needs to be focused. Implication of traditional and modern high-throughput biological, serological and molecular indexing techniques, such as ELISA, RT-PCR, PAGE, should be put in practice for the detection and indexing of virus and virus-like diseases of citrus plants. Moreover, citrus nurseries should be registered and indiscriminate multiplication and sale of uncertified citrus plants should be prohibited.

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