



Food and Agriculture  
Organization of the  
United Nations



**International Plant Protection Convention**  
Protecting the world's plant resources from pests

## DP 15: *Citrus tristeza virus*

This page is intentionally left blank

# ISPM 27

## Diagnostic protocols for regulated pests

### DP 15: *Citrus tristeza virus*

Adopted 2016; published 2017

#### CONTENTS

1. Pest Information .....	3
2. Taxonomic Information .....	4
3. Detection and Identification .....	4
3.1 Host range .....	4
3.2 Symptoms.....	5
3.3 Biological indexing .....	5
3.4 Sampling and sample preparation for serological and molecular testing.....	6
3.4.1 Sampling .....	6
3.4.2 Preparation of tissue prints.....	7
3.4.2.1 Preparation of tissue prints for serological testing .....	7
3.4.2.2 Preparation of tissue prints and aphid squashes for molecular amplification testing.....	7
3.4.3 Preparation of plant extracts for serological and molecular amplification testing .....	7
3.5 Serological tests .....	8
3.5.1 Direct tissue print-ELISA.....	8
3.5.2 DAS-ELISA .....	8
3.6 Molecular tests.....	9
3.6.1 RNA purification, immunocapture and cDNA synthesis .....	10
3.6.1.1 RNA purification.....	10
3.6.1.2 Immunocapture .....	10
3.6.1.3 cDNA synthesis.....	10
3.6.2 IC-RT-PCR .....	10
3.6.3 IC nested RT-PCR in a single closed tube .....	10
3.6.4 General considerations for RT-PCR and nested RT-PCR.....	11
3.6.5 Real-time RT-PCR .....	11
3.6.7 Interpretation of results from conventional and real-time RT-PCR.....	12
3.6.1 Controls for molecular tests .....	12
3.6.7.1 Conventional RT-PCR and IC-RT-PCR .....	13
3.6.7.2 Real-time RT-PCR.....	13
3.7 Validation by a test performance study .....	13
4. Identification of Aggressive CTV Strains .....	14
4.1 Biological indexing .....	15
4.2 Serological tests using MCA13.....	15

---

4.2.1	Direct tissue print-ELISA.....	15
4.2.2	DAS-ELISA .....	15
5.	Records .....	15
6.	Contact Points for Further Information .....	15
7.	Acknowledgements .....	16
8.	References .....	16
9.	Figures .....	19

## 1. Pest Information

*Citrus tristeza virus* (CTV) causes one of the most damaging diseases of citrus, devastating epidemics of which have changed the course of the citrus industry (Moreno *et al.*, 2008). The term “tristeza”, in Portuguese meaning “sadness” or “melancholy”, refers to the decline seen in many citrus species when grafted on *Citrus aurantium* (sour orange) or *Citrus limon* (lemon) rootstocks. Although tristeza is predominantly a bud union disease (Román *et al.*, 2004), some CTV strains induce other syndromes, including stem pitting, stunting, reduced productivity and impaired fruit quality of many commercial cultivars, even when they are grafted on tristeza-tolerant rootstocks.

CTV probably originated in Malaysia and other countries of Southeast Asia, the putative area of origin of citrus, and it has been disseminated to almost all citrus-growing countries through the movement of infected plant material. Subsequent local spread by aphid vector species has created major epidemics.

Tree losses on sour orange rootstock were first reported in South Africa in the early twentieth century, and in Argentina and Brazil in the 1930s, likely following the introduction of CTV-infected plants probably infested with the aphid vector most efficient for transmitting the virus, *Toxoptera citricida* Kirkaldy. CTV-induced tree decline has killed or rendered unproductive trees grafted on sour orange rootstock (Bar-Joseph *et al.*, 1989; Cambra *et al.*, 2000a). CTV outbreaks have been observed in the United States, some Caribbean countries and some Mediterranean countries (especially Italy and Morocco). CTV has affected an estimated 38 million trees in the Americas (mainly Argentina, Brazil, Venezuela and California (United States)), 60 million trees in the Mediterranean Basin (especially Spain, with about 50 million trees affected) and an estimated 5 million trees elsewhere, making a total of more than 100 million trees. Tristeza disease can be managed by using citrus rootstock species that induce tolerance to the tristeza disease. Some aggressive strains of CTV cause stem pitting in certain citrus cultivars regardless of the rootstock used. This has a significant impact on fruit quality and yield in several million trees infected with these aggressive strains in most citrus industries worldwide, with the exception of those in the Mediterranean Basin where aggressive strains are not present or are not predominant. To effectively manage the stem pitting disease some citrus industries have adopted a strategy of prophylactically inoculating trees with mild strains of CTV, otherwise known as cross-protection (Broadbent *et al.*, 1991; da Graça and van Vuuren, 2010).

CTV is the largest and most complex member of the genus *Closterovirus* (Moreno *et al.*, 2008). The virions are flexuous, filamentous, 2 000 nm in length and 11 nm in diameter, and contain a non-segmented, positive-sense, single-stranded RNA genome. The CTV genome contains 12 open reading frames (ORFs), encoding at least 17 proteins, and two untranslated regions (UTRs). ORFs 7 and 8 encode proteins with estimated molecular weights of 27.4 kDa (P27) and 24.9 kDa (P25) that have been identified as the capsid proteins. CTV diversity is greater than previously thought; new genotypes have diverged from the ancestral population or have arisen through recombination with previously described strains (Harper *et al.*, 2008). CTV populations in citrus trees are quasispecies in nature, so a complex mixtures of viral genotypes and defective viral RNAs developed during the long-term vegetative propagation of virus isolates through grafting and the mixing of such isolates with aphid-vectored isolates. This results in CTV isolates containing a population of sequence variants, with one usually being predominant (Moreno *et al.*, 2008).

CTV is readily transmitted experimentally by grafting healthy citrus with virus-infected plant material. It is naturally transmitted by certain aphid species in a semi-persistent manner. The most efficient vector of CTV worldwide is *T. citricida*. *T. citricida* is well established in Asia, Australia, sub-Saharan Africa, Central and South America, the Caribbean, Florida (United States) and northern mainland Spain and Portugal as well as the Madeira Islands (Ilharco *et al.*, 2005; Moreno *et al.*, 2008). However, *Aphis gossypii* Glover is the main vector in Spain, Israel, some citrus growing areas in California (United States) and in all locations where *T. citricida* is absent (Yokomi *et al.*, 1989; Cambra *et al.*, 2000a; Marroquín *et al.*, 2004). The comparative effects of aphid vector species on the spread of CTV have been reported (Gottwald *et al.*, 1997). Other aphid species have also been described as CTV vectors (Moreno *et al.*, 2008), including *Aphis spiraecola* Patch, *Toxoptera aurantii*

(Boyer de Fonsicolombe), *Myzus persicae* (Sulzer), *Aphis craccivora* Koch and *Uroleucon jaceae* (Linnaeus). Although these listed aphid species were shown to be less efficient vectors of CTV than *T. citricida* and *A. gossypii* in experimental transmission studies, they are the predominant aphid species in some areas and are therefore likely to play a role in CTV spread, compensating for their poor transmission efficiency by their abundance (Marroquín *et al.*, 2004).

The spatial and temporal spread of CTV in citrus orchards has been studied in different parts of the world (Gottwald *et al.*, 2002). These studies provide evidence for the fact that a long period of time may elapse between the introduction of a primary source of CTV inoculum and the development of a tristeza disease epidemic (Garnsey and Lee, 1988).

## 2. Taxonomic Information

**Name:** *Citrus tristeza virus* (acronym CTV)

**Synonyms:** Tristeza virus

**Taxonomic position:** *Closteroviridae*, *Closterovirus*

**Common names:** Tristeza virus, citrus tristeza virus

## 3. Detection and Identification

Detection and identification of CTV can be achieved using biological, serological or molecular amplification tests (Figure 1 and Figure 2). The use of any one of these tests is the minimum requirement to detect and identify CTV (i.e. during routine diagnosis of the pest when it is widely established in a country). In instances where the national plant protection organization (NPPO) requires additional confidence in the identification of CTV (i.e. detection in an area where the virus is not known to occur or detection in a consignment originating from a country where the pest is declared to be absent), further tests should be done. Where the initial identification was done using a molecular amplification test, subsequent tests should be serological, and vice versa. Further tests may also be done to identify the strain of CTV present, in which case sequencing of the amplicon produced by polymerase chain reaction (PCR) may be needed. In all cases, for the tests to be considered valid, positive and negative controls must be included. The recommended techniques for the biological, serological and molecular amplification tests are described in the following sections. A flow chart for strain identification of CTV is presented in Figure 2.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

### 3.1 Host range

Under natural conditions, CTV readily infects most species of *Citrus* and *Fortunella* and some species in genera known as citrus-relatives of the family Rutaceae that are also susceptible hosts of CTV; namely, *Aegle*, *Aeglopsis*, *Afraegle*, *Atalantia*, *Citropsis*, *Clausena*, *Eremocitrus*, *Hesperatus*, *Merrillia*, *Microcitrus*, *Pamburus*, *Pleiospermium* and *Swinglea* (Duran-Vila and Moreno, 2000; Timmer *et al.*, 2000). Most *Poncirus trifoliata* (trifoliate orange) clones and many of their hybrids as well as *Fortunella crassifolia* (Meiwa kumquat) and some *Citrus grandis* (pomelo) are resistant to most CTV strains (Moreno *et al.*, 2008). Consequently, CTV is absent or in very low concentration in these species. *Citrus reticulata* (mandarin), *Citrus sinensis* (sweet orange) and *Citrus latifolia* (lime) are among the cultivars most susceptible to natural CTV infection, followed by *Citrus paradisi* (grapefruit), *Citrus unshiu* (Satsuma mandarin) and *C. limon* cultivars. Among the species used as

rootstock, *Citrus macrophylla* (alemow), *Citrus volkameriana* (Volkamer lemon), *Citrus reshni* (Cleopatra mandarin) and *Citrus limonia* (Rangpur lime or lemandarin) are highly susceptible to natural CTV infection, whereas Carrizo and Troyer citranges (hybrids of sweet orange and trifoliate orange) and *C. aurantium* are rarely infected. *P. trifoliata* and *C. paradisi* × *P. trifoliata* (citrumelo) rootstocks are resistant to most CTV strains. *Passiflora gracilis* and *Passiflora coerulea* are experimental non-citrus hosts.

### 3.2 Symptoms

Symptom expression in CTV-infected citrus hosts is highly variable and is affected by environmental conditions, host species and the aggressiveness of the CTV strain. In addition, the virus may remain latent for several years. Some CTV strains are mild and produce no noticeable effects on most commercial citrus species, including citrus grafted on *C. aurantium*. In general, mandarins are especially tolerant to CTV infection. *C. sinensis*, *C. aurantium* (as a seedling and not as grafted rootstock), *Citrus jambhiri* (rough lemon) and *C. limonia* are usually symptomless when infected but may react to some aggressive strains. Citrus hosts that manifest symptoms are likely to include lime, grapefruit, some cultivars of pomelo, alemow and sweet orange, some citrus hybrids and some citrus-relatives of the family Rutaceae mentioned in section 3.1.

Depending on the CTV strain and citrus species or scion–rootstock combination, the virus may cause no symptoms or one of three syndromes: tristeza; stem pitting; or seedling yellows, which is mainly seen under greenhouse conditions. These three syndromes are described in the paragraphs below. Figure 1 shows the main symptoms caused by CTV.

One of the most economically significant outcomes of CTV infection is tristeza (a bud union disease), which is characterized by the decline of trees grafted on sour orange or lemon rootstocks. Sweet orange, mandarin and grapefruit scions on these rootstocks become stunted, chlorotic and often die after a period of several months or years (i.e. they experience a slow decline), while other scions experience a rapid decline or collapse some days after the first symptom is observed. The decline results from the physiological effects of the virus on the phloem of the susceptible rootstock just below the bud union. Trees that decline slowly generally have a bulge above the bud union, a brown line just at the point of bud union, and inverse pinhole pitting (honeycombing) on the inner face of sour orange rootstock bark. Stunting, leaf cupping, vein clearing, chlorotic leaves, stem pitting and reduced fruit size are common symptoms observed on susceptible hosts. However some isolates of the virus, particularly in the Mediterranean Basin citrus industry, do not induce decline symptoms until many years after infection, even in trees grafted on sour orange.

Aggressive CTV strains can severely affect trees, inducing stem pitting on the trunk and branches of lime, grapefruit and sweet orange. Stem pitting may sometimes cause a bumpy or ropy appearance of the trunks and limbs of adult trees, deep pits in the wood under depressed areas of the bark, and a reduction in fruit quality and yield. Alemow rootstocks are seriously affected by most of all CTV strains as the rootstock develops stem pitting that results in reduced tree vigour.

The seedling yellows syndrome is characterized by stunting, production of chlorotic or pale leaves, development of a reduced root system, and cessation of growth of trees grafted on sour orange, grapefruit and lemon seedlings cultivated under greenhouse conditions (20–26°C).

### 3.3 Biological indexing

The objective of biological indexing is to detect the presence of CTV in plant accessions or selections or in samples whose sanitary status is being assessed, and to estimate the aggressiveness of the isolate on *Citrus aurantifolia* (Mexican, key or Omani lime), *C. macrophylla* or *Citrus paradisi* Macfadyen (Duncan grapefruit) seedlings. The indicator is a graft inoculated according to conventional methods and held under standard conditions (Roistacher, 1991), with four to six replicates (or with two to three replicates if sufficient samples cannot be taken). Vein clearing in young leaves, leaf cupping or leaf distortion, short internodes, stem pitting or seedling yellows symptoms on these sensitive indicator

plants are each evidence of CTV infection after graft inoculation. Symptom onset is compared against that on positive and negative control plants. Illustrations of symptoms caused by CTV on indicator plants can be found in Roistacher (1991) and Moreno *et al.* (2008).

Biological indexing is used widely in certification schemes, as it is considered a sensitive and reliable method for the detection of a new or an unusual strain of the virus. However, it has some disadvantages: it is not a rapid test (symptom development requires three to six months post-inoculation); it can only be used to test budwood; it requires dedicated facilities such as temperature-controlled insect-proof greenhouse space; and it requires dedicated staff who can grow healthy and vigorous indicator host plants that will show appropriate symptoms and experienced staff who can accurately interpret observed disease symptoms that can be confused with symptoms of other graft-transmissible agents. Moreover, asymptomatic CTV strains that do not induce symptoms (latent strains) are not detectable on indicator plants (e.g. the CTV strain K described by Albertini *et al.* (1988)).

There are few quantitative data published on the specificity, sensitivity, other diagnostic parameters and reliability of biological assays by grafting indicator plants (indexing) for CTV detection, diagnosis or identification. Cambra *et al.* (2002) in the European Diagnostic Protocols project (DIAGPRO) and Vidal *et al.* (2012) compared Mexican lime indexing with direct tissue print-enzyme-linked immunosorbent assay (ELISA) (section 3.5.1) (using 3DF1 + 3CA5 monoclonal antibodies) and tissue print real-time reverse transcription (RT)-PCR (section 3.6.5) and concluded that either laboratory method can accurately substitute for the conventional Mexican lime biological indexing for CTV detection.

### 3.4 Sampling and sample preparation for serological and molecular testing

#### 3.4.1 Sampling

General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*) and in Cambra *et al.* (2002) specifically for CTV sampling. Appropriate sampling is crucial for CTV detection and identification by biological, serological or molecular amplification methods. Changes to an accepted sampling scheme could result in an effective diagnostic protocol generating false positive or false negative results. The standard sample for adult trees is five young shoots or fruit peduncles, ten fully expanded leaves, or five flowers or fruits collected around the canopy of each individual tree from each scaffold branch. Samples (shoots or fully expanded leaves and peduncles) can be taken at any time of the year from sweet orange, mandarin, lemon and grapefruit in temperate Mediterranean climates, but spring and autumn are the optimal sampling periods in tropical and subtropical climates for achieving high CTV titres. In these climates, a reduced CTV titre is observed in Satsuma mandarin during summer; consequently, the recommended period for sampling includes all vegetative seasons, with the exception of hot days (35–40 °C) in summer. Roots, however, can be sampled during hot periods if required. Flowers or fruits (when available) are also suitable materials for sampling (Cambra *et al.*, 2002). Tissue from the fruit peduncle in the region of the albedo, where the peduncle is joined to the fruit, or from the columela is the most suitable fruit sample. Standard requirements for sampling nursery plants include the collection of two young shoots or four leaves per plant. Usually chip non-budding (small pieces of bark without buds) or even leaves from infected plants are collected at any time of the year (but preferably during the vegetative period) from at least one year old shoots or tree branches for indexing according to Roistacher (1991).

Shoots, leaf petioles, fruit peduncles and flowers can be stored at approximately 4 °C for up to seven days before processing. Fruits can be stored for one month at approximately 4 °C. Use beyond these time frames may result in lower titres and the potential for false negative results in diagnostic methods

Composite samples, to be tested as a single sample, can be collected together (usually two leaves or one shoot from one to ten nursery plants or ten leaves or five shoots per adult trees collected around



the canopy) for serological or molecular amplification tests. In some circumstances (e.g. routine screening for CTV widely established in a country or an area), multiple plants may be tested simultaneously using a composite sample derived from a number of plants. The decision to test individual plant or composite plant samples by serological or molecular amplification methods depends on the virus concentration in the plants, the expected prevalence of CTV in the area (Vidal *et al.*, 2012), the limit of detection of the test method to be used, and the level of confidence required by the NPPO.

Aphids (fresh or preserved in 70% alcohol) can be individually tested for the presence of CTV. The aphids are collected directly from established colonies or caught in traps: suction traps, classic Moericke yellow water traps or the sticky shoot trap are recommended. Specimens collected are used preferably for squash real-time RT-PCR (Bertolini *et al.*, 2008) or other molecular amplification tests (Marroquín *et al.*, 2004).

### **3.4.2 Preparation of tissue prints**

#### **3.4.2.1 Preparation of tissue prints for serological testing**

Tender shoots, leaf petioles, fruit peduncles or flower ovaries are cut cleanly. The freshly cut sections are carefully pressed against a nitrocellulose or ester of cellulose membrane (0.45 mm) and the trace or print is allowed to dry for 2–5 min. For routine serological testing, at least two prints should be made per selected shoot (one from each end of the shoot) or peduncle and one per leaf petiole or flower ovary. Printed membranes can be kept for several months in a dry and dark place

#### **3.4.2.2 Preparation of tissue prints and aphid squashes for molecular amplification testing**

Collection of plant material by hand is recommended to avoid contamination of samples by scissors. Tender shoots with fully expanded leaves or mature leaves are collected around the canopy of the tree. The leaf petiole of two leaves or shoots is pressed directly on Whatman<sup>1</sup> 3MM paper (0.45 mm) or positively charged nylon membrane. Several partially overlapping imprints from different leaves are made on approximately 0.5 cm<sup>2</sup> of the paper or membrane, according to Bertolini *et al.* (2008). The trace or print is allowed to dry for 2–5 min. For routine molecular amplification testing, one print should be made per selected leaf pedicel. Individual aphids are squashed directly onto Whatman<sup>1</sup> 3MM paper or positively charged nylon membrane with the aid of the round bottom of an Eppendorf<sup>1</sup> tube to achieve total disruption of the specimen (Bertolini *et al.*, 2008). Printed or squashed membranes can be kept for several months in a dry and dark place.

Direct methods of sample preparation (tissue print or squash) without extract preparation have been validated as an alternative to conventional extract preparation for sample processing (Vidal *et al.*, 2012).

### **3.4.3 Preparation of plant extracts for serological and molecular amplification testing**

Fresh plant material, 0.2–0.5 g, is cut in small pieces with disposable razor blades or bleach-treated scissors to avoid sample to sample contamination and placed in a suitable tube or plastic bag. Extracts for serological testing can be prepared in tubes or in plastic bags. Samples for molecular amplification testing should only be prepared in individual plastic bags to avoid contamination among samples. The sample is homogenized thoroughly in 4–10 ml (1:20 w/v, unless otherwise stated by the manufacturer) extraction buffer using an electrical tissue homogenizer, a manual roller, a hammer or a similar tool.

---

<sup>1</sup> In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

The extraction buffer is phosphate-buffered saline (PBS), pH 7.2–7.4 (NaCl<sub>2</sub>, 8 g; KCl, 0.2 g; Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 2.9 g; KH<sub>2</sub>PO<sub>4</sub>, 0.2 g; distilled water, 1 litre) supplemented with 0.2% sodium diethyl dithiocarbamate (DIECA) or 0.2% mercaptoethanol, or an alternative suitably validated buffer.

### 3.5 Serological tests

ELISA using validated monoclonal antibodies or polyclonal antibodies is highly recommended for screening large numbers of samples for CTV detection and identification. The production of monoclonal antibodies specific to CTV (Vela *et al.*, 1986; Permar *et al.*, 1990) and others reviewed by Nikolaeva *et al.* (1996) solved the problem of the diagnostic specificity presented by polyclonal antibodies (Cambra *et al.*, 2011) and thus increased the diagnostic sensitivity of serological tests. A mixture of the two monoclonal antibodies 3DF1 and 3CA5, or their recombinant versions (Terrada *et al.*, 2000), recognizes all CTV isolates tested from different international collections (Cambra *et al.*, 1990). A detailed description, characterization and validation of these monoclonal antibodies is provided in Cambra *et al.* (2000a). A mixture of the monoclonal antibodies 4C1 and 1D12 produced in Morocco is reported to react against a broad spectrum of CTV strains (Zebzami *et al.*, 1999), but there are no validation data available.

#### 3.5.1 Direct tissue print-ELISA

Direct tissue print-ELISA, also referred to as immunoprinting ELISA or direct tissue blot immunoassay (DTBIA), is performed according to Garnsey *et al.* (1993) and Cambra *et al.* (2000b) using the method described below. A complete kit (validated in test performance and in several published studies) based on CTV-specific 3DF1 + 3CA5 monoclonal antibodies (Vela *et al.*, 1986), including preprinted membranes with positive and negative controls and all reagents, buffers and substrate, is available from Plant Print Diagnostics SL<sup>1</sup>. A similar but non-validated kit based on Zebzami *et al.*, (1999) 4C1 and 1D12 antibodies is available from Agdia<sup>1</sup>.

Membranes that have been tissue printed (recommended size: approximately 7 × 13 cm) are placed in an appropriate container (tray, hermetic container or plastic bag), covered with a 1% solution of bovine serum albumin (BSA) in distilled water and incubated for 1 h at room temperature or overnight (about 16 h) at 4 °C (the latter is recommended). Slight agitation is beneficial during this step. The BSA solution is discarded but the membranes are kept in the same container. A conjugate solution is prepared that consists of equal concentrations of CTV-specific 3DF1 + 3CA5 monoclonal antibodies linked to alkaline phosphatase (approximately 0.1 µg/ml of each monoclonal antibody in PBS) or of 3DF1 scFv-AP/S + 3CA5 scFv-AP/S fusion proteins expressed in *Escherichia coli* (an appropriate dilution in PBS) (Terrada *et al.*, 2000). The conjugate solution is poured onto the membranes, covering them, and the membranes are incubated for 3 h at room temperature, with slight agitation. The conjugate solution is then discarded. The membranes and the container are rinsed with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20), and washed by shaking (manually or mechanically) for 5 min. The washing buffer is discarded and the washing process is repeated twice. The substrate for alkaline phosphatase (Sigma<sup>1</sup> Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets, following the manufacturer's gives to a final concentration of 0.33 mg/ml NBT and 0.175 mg/ml BCIP) is then poured over the membranes and the membranes are incubated until a purple-violet colour appears in the positive controls (about 10–15 min). The reaction is stopped by washing the membranes with tap water. The membranes are spread on absorbent paper and allowed to dry. The prints are examined using a low-power magnification (×10 to ×20). The presence of purple-violet precipitates in the vascular region of plant material reveals the presence of CTV.

#### 3.5.2 DAS-ELISA

Double antibody sandwich (DAS)-ELISA is performed according to Garnsey and Cambra (1991) using the method described below. Complete kits based on validated CTV-specific 3DF1 + 3CA5 monoclonal antibodies (Plant Print Diagnostics SL<sup>1</sup>) and on different polyclonal antibodies (Agdia<sup>1</sup>, Agritest<sup>1</sup>, Bioreba<sup>1</sup>, Loewe<sup>1</sup>, Sediag<sup>1</sup>) are available.

Two wells of a microtiter plate are used for each sample and at least two wells for positive and negative controls. An appropriate dilution is prepared of the polyclonal or monoclonal (3DF1 + 3CA5) antibodies (usually 1–2 µg/ml total immunoglobulins) in carbonate buffer, pH 9.6 (Na<sub>2</sub>CO<sub>3</sub>, 1.59 g; NaHCO<sub>3</sub>, 2.93 g; distilled water, 1 litre), and 200 µl is added to each well. The plate is incubated for 4 h at 37 °C or overnight (about 16 h) at 4 °C. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). The plant extract (section 3.4.3) is then added, 200 µl to each well. After incubation for 16 h at 4 °C, the plates are washed three times as described for direct tissue print-ELISA (section 3.5.1). Specific polyclonal or monoclonal (3DF1 + 3CA5) antibody mixtures linked with alkaline phosphatase are prepared at appropriate dilutions (approximately 0.1 µg/ml in PBS with 0.5% BSA) then 200 µl is added to each well. Incubation is carried out for 3 h at 37 °C. The plates are again washed as described for direct tissue print-ELISA (section 3.5.1). A solution of 1 mg/ml alkaline phosphatase (p-nitrophenyl phosphate) in substrate buffer (97 ml diethanolamine in 800 ml distilled water, pH adjusted to 9.8 with concentrated HCl, and the total volume then made up to 1 000 ml with distilled water) is prepared and 200 µl is added to each well. The plates are incubated at room temperature and read at 405 nm at regular intervals within 120 min, or following the instructions of the supplier of the polyclonal antibody being used.

The ELISA is considered negative if the average optical density (OD) value from each of the duplicate sample wells is <0.1 or is <2× the OD value of the negative control of healthy plant extracts. The ELISA is considered positive if the average OD value from each of the duplicate sample wells is ≥2× the OD value of the negative control of healthy plant extracts. When using polyclonal antibodies, it is essential that the negative controls are as similar as possible to the matrix tested in the same plate.

The method using 3DF1 + 3CA5 monoclonal antibodies was validated in a DIAGPRO ring test (Cambra *et al.*, 2002). A comparison of that method with other techniques and the diagnostic parameters are given in section 3.7.

While some mixtures of monoclonal antibodies detect all CTV strains specifically, sensitively and reliably, some polyclonal antibodies are not specific and have limited sensitivity (Cambra *et al.*, 2011). Therefore, the use of additional methods is recommended in situations where polyclonal antibodies have been used in an assay and the NPPO requires additional confidence in the identification of CTV.

### 3.6 Molecular tests

After the complete nucleotide sequence of the CTV genomic RNA became available, various diagnostic procedures based on specific detection of viral RNA were developed, including molecular hybridization with complementary (c)DNA or cRNA probes and several methods based on RT-PCR (Moreno *et al.*, 2008). These RT-PCR-based methods have greatly improved the sensitivity of detection, allowing quantification of viral RNA copies in infected citrus tissue or in CTV-viruliferous aphid species (Bertolini *et al.*, 2008). The use of a high throughput technique such as real-time RT-PCR avoids the need for any post-amplification processing (e.g. gel electrophoresis) and is therefore quicker and has less opportunity for cross-contamination than conventional PCR.

With the exception of immunocapture (IC)-RT-PCR (for which RNA isolation is not required), RNA extraction should be done using appropriately validated protocols. The samples should be placed in individual plastic bags to avoid cross-contamination during extraction. Alternatively, spotted plant extracts, printed tissue sections or squashes of plant material can be immobilized on blotting paper or nylon membranes and analysed by real-time RT-PCR (Bertolini *et al.*, 2008). It is not recommended to use spotted or tissue printed samples in conventional PCR because of its lower sensitivity compared with real-time RT-PCR, can result in false negatives.

### 3.6.1 RNA purification, immunocapture and cDNA synthesis

#### 3.6.1.1 RNA purification

RNA purification should be done using appropriately validated protocols or using an RNA purification kit according to the manufacturer's instructions. The extracted RNA should be stored at  $-70^{\circ}\text{C}$  (preferably) or at  $-20^{\circ}\text{C}$  until its use as a template and for less than one year. Storage should be in small quantities to avoid degradation of RNA due to repeated freeze–thaw cycles.

#### 3.6.1.2 Immunocapture

Immunocapture is an alternative option to RNA purification. For this procedure, a diluted antibody mixture is prepared, consisting of  $1\text{ }\mu\text{g/ml}$  CTV-specific polyclonal antibodies or a dilution of monoclonal antibodies (3DF1 + 3CA5,  $0.5\text{ }\mu\text{g/ml}$  +  $0.5\text{ }\mu\text{g/ml}$ ) in carbonate buffer, pH 9.6 (see section 3.5.2 for the composition of carbonate buffer). The antibody mixture is then dispensed into microtubes ( $100\text{ }\mu\text{l}$  per tube) and the tubes are incubated for 3 h at  $37^{\circ}\text{C}$ . The coated tubes are washed twice with  $150\text{ }\mu\text{l}$  sterile washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20; see section 3.4.3 for the composition of PBS). Plant extract ( $100\text{ }\mu\text{l}$ ) could optionally be clarified by centrifugation or filtration through filter paper or directly used as crude extract and aliquots are dispensed into the antibody-coated microtubes. The tubes are incubated for a minimum of 2 h on ice or alternatively for 2 h at  $37^{\circ}\text{C}$ . After this immunocapture phase, the microtubes are washed three times with  $150\text{ }\mu\text{l}$  sterile washing buffer. It is in these washed tubes that cDNA synthesis and PCR amplification are performed.

#### 3.6.1.3 cDNA synthesis

Because the preservation of RNA during storage is problematic, it is recommended to synthesize cDNA, which can be preserved for long periods with minimal temperature requirements compared with RNA. Several commercial kits are available for cDNA synthesis.

### 3.6.2 IC-RT-PCR

According to Olmos *et al.* (1999) the primers are:

PIN1: 5'-GGT TCA CGC ATA CGT TAA GCC TCA CTT-3'

PIN2: 5'-TAT CAC TAG ACA ATA ACC GGA TGG GTA -3'

The RT-PCR mixture consists of: ultrapure water,  $14.3\text{ }\mu\text{l}$ ;  $10\times$  Taq DNA polymerase buffer,  $2.5\text{ }\mu\text{l}$ ;  $25\text{ mM}$   $\text{MgCl}_2$ ,  $1.5\text{ }\mu\text{l}$ ;  $5\text{ mM}$  dNTPs,  $1.25\text{ }\mu\text{l}$ ; 4% Triton X-100,  $2\text{ }\mu\text{l}$ ;  $25\text{ }\mu\text{M}$  primer PIN1,  $1\text{ }\mu\text{l}$ ;  $25\text{ }\mu\text{M}$  primer PIN2,  $1\text{ }\mu\text{l}$ ; dimethyl sulfoxide (DMSO),  $1.25\text{ }\mu\text{l}$ ;  $10\text{ U}/\mu\text{l}$  AMV reverse transcriptase,  $0.1\text{ }\mu\text{l}$ ; and  $5\text{ U}/\mu\text{l}$  Taq DNA polymerase,  $0.1\text{ }\mu\text{l}$ . Reaction mixture ( $25\text{ }\mu\text{l}$ ) is added directly to the washed antibody-coated microtubes. The cycling parameters for RT-PCR are:  $42^{\circ}\text{C}$  for 45 min and  $92^{\circ}\text{C}$  for 2 min followed by 40 cycles of ( $92^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 1 min), with a final elongation step at  $72^{\circ}\text{C}$  for 10 min followed by cooling at  $8^{\circ}\text{C}$ . The expected amplicon size is 131 base pairs (bp).

The method was validated in a DIAGPRO ring test (Cambra *et al.*, 2002). A comparison with other techniques and the diagnostic parameters are given in section 3.7.

### 3.6.3 IC nested RT-PCR in a single closed tube

According to Olmos *et al.* (1999) the primers are:

PEX1: 5'-TAA ACA ACA CAC ACT CTA AGG-3'

PEX2: 5'-CAT CTG ATT GAA GTG GAC-3'

PIN1: 5'-GGT TCA CGC ATA CGT TAA GCC TCA CTT-3'

PIN2: 5'-TAT CAC TAG ACA ATA ACC GGA TGG GTA-3'

The device for compartmentalization of a 0.5 ml microtube for nested RT-PCR in a single closed tube is according to Olmos *et al.* (1999). The RT-PCR master mix consists of two reaction mixtures:

**A** (dropped in the bottom of the microtube): ultrapure water, 15.8 µl; 10× Taq DNA polymerase buffer, 3 µl; 25 mM MgCl<sub>2</sub>, 3.6 µl; 5 mM dNTPs, 2 µl; 4% Triton X-100, 2.2 µl; 25 µM primer PEX1, 0.6 µl; 25 µM primer PEX2, 0.6 µl; DMSO, 1.5 µl; 10 U/µl AMV reverse transcriptase, 0.2 µl; and 5 U/µl Taq DNA polymerase, 0.5 µl.

**B** (placed in the cone): ultrapure water, 2.6 µl; 10× Taq DNA polymerase buffer, 1 µl; 25 µM primer PIN1, 3.2 µl; and 25 µM primer PIN2, 3.2 µl.

The cycling parameters for RT-PCR are: 42 °C for 45 min and 92 °C for 2 min followed by 25 cycles of (92 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min). After this first step, the tube is vortexed and centrifuged (6000 r.p.m. for 5 s) to mix B with the products of the first amplification. The tube is then placed in the thermal cycler again and the reaction proceeds as follows: 40 cycles of (92 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min), with a final elongation step at 72 °C for 10 min followed by cooling at 8 °C. The expected amplicon size is 131 bp.

The method was validated in a DIAGPRO ring test (Cambra *et al.*, 2002). A comparison with other techniques and the diagnostic parameters are given in section 3.7.

### 3.6.4 General considerations for RT-PCR and nested RT-PCR

The RT-PCR protocols may need to be modified and optimized when using different reagents or thermocycler platforms.

If conventional RT-PCR is used for the detection of CTV, IC-RT-PCR is recommended. Conventional RT-PCR without IC is not sensitive, and may give false negative results. It is possible that the presence of inhibitors affects the sensitivity of conventional RT-PCR.

The test on a sample is negative if the CTV-specific amplicon of the expected size is not detected in the sample in question but is detected in all positive controls. The test on a sample is positive if the CTV-specific amplicon of the expected size is detected in the sample in question, providing that there is no amplification in any of the negative controls.

### 3.6.5 Real-time RT-PCR

Two real-time RT-PCR assays have been described, one by Bertolini *et al.* (2008) and the other by Saponari *et al.* (2008).

According to Bertolini *et al.* (2008) the primers and probe are:

3'UTR1: 5'-CGT ATC CTC TCG TTG GTC TAA GC-3'

3'UTR2: 5'-ACA ACA CAC ACT CTA AGG AGA ACT TCT T-3'

181T: FAM-TGG TTC ACG CAT ACG TTA AGC CTC ACT TG-TAMRA

The reaction is carried out in a final volume of 25 µl. The real-time RT-PCR mixture consists of: ultrapure water, 0.95 µl; 2× AgPath-ID One-Step RT-PCR Master Mix (Applied Biosystems<sup>1</sup>), 12.5 µl; 25× RT-PCR enzyme mix, 1 µl; 10 µM primer 3'UTR1, 2.4 µl; 10 µM primer 3'UTR2, 2.4 µl; 5 µM probe FAM-labelled 181T, 0.75 µl; and 5 µl of RNA extracted or released from a membrane added to 20 µl of the real-time RT-PCR mix. The cycling parameters are: 45 °C for 10 min and 95 °C for 10 min followed by 45 cycles of (95 °C for 15 s and 60 °C for 1 min). The expected amplicon size is 95 bp.

For the tissue print real-time RT-PCR, a diagnostic sensitivity of 0.98, a specificity of 0.85, and a positive and negative likelihood ratio of 6.63 and 0.021, respectively, were estimated (Vidal *et al.*,



2012). These diagnostic parameters show that tissue print real-time RT-PCR was the most sensitive technique when compared with direct tissue print-ELISA, validating its use for routine CTV detection and diagnosis, and highly recommending it for assessing the CTV-free status of any plant material. The high sensitivity of this technique allows the accurate analysis of composite samples (up to ten batched trees or nursery plants) as one diagnostic sample when tested in any season of the year, and it also allows analysis of aphid species to detect low concentrations of CTV. For additional diagnostic parameters of validation of tissue print real-time RT-PCR, see section 3.7.

According to Saponari *et al.* (2008) the primers and probe are:

P25F: 5'-AGC RGT TAA GAG TTC ATC ATT RC-3'

P25R: 5'-TCR GTC CAA AGT TTG TCA GA-3'

CTV-CY5: CY5-CRC CAC GGG YAT AAC GTA CAC TCG G

The reaction is carried out in a final volume of 25 µl. The real-time RT-PCR mixture consists of: ultrapure water, 6.6 µl; 2× iScript One-Step RT-PCR Kit for Probes (Bio-Rad), 12.5 µl; iScript reverse transcriptase supermix, 0.5 µl; 10 µM primer P25F, 1 µl; 10 µM primer P25R, 2 µl; 5 µM probe CTV-CY5, 0.4 µl; and 2 µl of RNA extracted or released from a membrane added to 23 µl of the real-time RT-PCR mix. The cycling parameters are: 55 °C for 2 min and 95 °C for 5 min followed by 40 cycles of (95 °C for 15 s and 59 °C for 30 s). The expected amplicon size is 101 bp.

Diagnostic parameters (i.e. sensitivity, specificity, accuracy, positive and negative likelihood ratios and post-test probability of disease) are not reported for this real-time RT-PCR protocol.

### 3.6.7 Interpretation of results from conventional and real-time RT-PCR

#### 3.6.1 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For RT-PCR a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used

**Positive nucleic acid control.** This control is used to monitor the efficiency of the test method (apart from the extraction) and in RT-PCR, the amplification. Pre-prepared (stored) RNA or CTV-infected plant material printed on a membrane may be used. The stored RNA or CTV preparations should be verified periodically to determine the quality of the control with increased storage time.

**Internal control.** For the real-time RT-PCR described by Saponari *et al.* (2008), mRNA of the mitochondrial gene *NADH dehydrogenase 5* (*nad5*) could be incorporated into the RT-PCR protocol as an internal control to eliminate the possibility of RT-PCR false negatives due to nucleic acid extraction failure or degradation or the presence of RT-PCR inhibitors. Because this is a host target, care should be taken not to contaminate the laboratory with *nad5* DNA, which would result in false confidence in the internal control reaction.

**Negative amplification control (no template control).** This control is necessary for conventional and real-time RT-PCR to rule out false positives due to contamination during the preparation of the reaction mixture. RNase-free PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

**Positive extraction control.** This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality for RT-PCR and that the target virus is detectable. Nucleic acid is extracted from infected host tissue or healthy plant or insect tissues that have been spiked with CTV.

For RT-PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples.

**Negative extraction control.** This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended to be included when large numbers of positive samples are expected.

### 3.6.7.1 Conventional RT-PCR and IC-RT-PCR

The pathogen-specific RT-PCR will be considered valid only if:

- (1) the positive control produces the correct size amplicon for the virus; and
- (2) the negative extraction control and the negative amplification control do not produce amplicons of the correct size for the virus.

If the mRNA mitochondrial gene *nad5* internal control primers are also used (forward: 5'-GAT GCT TCT TGG GGC TTC TTG TT-3', reverse: 5'-CTC CAG TCA CCA ACA TTG GCA TAA-3'; 181 bp product), then the negative extraction control (healthy plant tissue) (if used), positive control and each of the test samples must produce a 115 bp amplicon. Failure of the samples to amplify with the internal control primers suggests for example that the RNA extraction has failed, RNA has not been included in the reaction mix, compounds inhibitory to RT-PCR are present in the RNA extract or the RNA has degraded.

The test on a sample will be considered positive if it produces an amplicon of the correct size.

### 3.6.7.2 Real-time RT-PCR

The pathogen-specific real-time RT-PCR will be considered valid only if:

- (1) the positive control produces an amplification curve with the virus-specific primers; and
- (2) the negative extraction control and the negative amplification control do not produce amplification curves with the virus-specific primers.

The test on a sample will be considered positive if it produces a typical amplification curve in an exponential manner. The cycle threshold (Ct) value needs to be verified in each laboratory when implementing the test for the first time.

## 3.7 Validation by a test performance study

In a DIAGPRO ring test (Cambra *et al.*, 2002) conducted by ten laboratories using a set of ten coded samples including CTV-infected and healthy tissue samples from the Valencian Institute of Agrarian Research (IVIA) collection, direct tissue print-ELISA using 3DF1 + 3CA5 monoclonal antibodies was 99% accurate (the number of true positives and true negatives diagnosed by the technique/number of samples tested). This accuracy was greater than that achieved with DAS-ELISA (98% accurate), IC-RT-PCR (94% accurate) and IC nested RT-PCR in a single closed tube (89% accurate). The sensitivity of direct tissue print-ELISA was 0.98 while the sensitivity of the other above-mentioned techniques was 0.96, 0.96 and 0.93, respectively (Vidal *et al.*, 2012). The diagnostic specificity of direct tissue print-ELISA was 1.0 while the diagnostic specificity of the other techniques was 1.0, 0.91 and 0.82, respectively. The positive predictive value (positive tests that actually have the disease; Sackett *et al.*, 1991) of direct tissue print-ELISA was 1.0 while the positive predictive value of the other techniques was 1.0, 0.94 and 0.89, respectively. The negative predictive value (Sackett *et al.*, 1991) of direct tissue print-ELISA was 0.97 while the negative predictive value of the other techniques was 0.95, 0.94 and 0.88, respectively (Harju *et al.*, 2000).

Direct tissue print-ELISA using 3DF1 + 3CA52 monoclonal antibodies was found to be the most reliable, simple and economical method for routine analysis of plant material when compared with biological indexing on Mexican lime, ELISA, IC-RT-PCR and IC nested RT-PCR for CTV detection (Cambra *et al.*, 2002). Direct tissue print-ELISA was also validated by Ruiz-García *et al.* (2005) and analysed by them to show that it was as sensitive as DAS-ELISA (the system detected 97% of positive

trees using four petioles) but was more user-friendly and less expensive. Direct tissue print-ELISA using 3DF1 + 3CA52 monoclonal antibodies was compared with biological indexing on Mexican lime and tissue print real-time RT-PCR for CTV detection (Vidal *et al.*, 2012). Various diagnostic parameters were evaluated and direct tissue print-ELISA was determined to be the most specific and accurate method, with the highest post-test probability of detecting the disease at any level of CTV prevalence.

#### 4. Identification of Aggressive CTV Strains

The identification of CTV strains requires a biological, serological or molecular amplification test.

There are no nucleic acid-based methods allowing reliable typing of CTV strains according to their aggressiveness because CTV is a phenotype. The genetic basis of the high biological variability of CTV is still largely unknown (Moreno *et al.*, 2008). Little is also known about the biological role of its diversity and particularly about the effects of recombination. Additionally, genotype grouping has not been standardized (Harper, 2013). A wide range of molecular methods have been used to differentiate between different CTV strains, including molecular hybridization, double-stranded (ds)RNA patterns, restriction fragment analyses of amplified CTV cDNA, amplification by PCR of different genome regions, real-time PCR (Moreno *et al.*, 2008; Yokomi *et al.*, 2010), genome sequencing, and re-sequencing microarrays. More recently, sequential analyses of enzyme immunoassays and capillary electrophoresis-single-strand conformation polymorphism have been attempted (Licciardello *et al.*, 2012). However, none of these technologies is practical for the reliable categorization of naturally spreading CTV strains, and none has been validated yet, their application being limited to research purposes.

Given the genetic and biological variability of CTV, techniques other than sequencing may provide erroneous results when attempting to identify CTV strains. The use of deep sequencing, also referred to as next generation sequencing, could rapidly supply information about the genomic sequence. However, the nucleotide sequence of CTV cannot yet be related to the biological properties and behaviour of the strain (i.e. aggressiveness and transmissibility). Even though CTV strains have been classified and grouped by their phenotype, virulence, host range, epitope composition and, more recently, by sequence identity of one or more genes (Moreno *et al.*, 2008), no clear correlation with biological behaviour has been found (Harper, 2013).

The recommended methods to obtain information related to the biological properties of a specific CTV strain are (Figure 2):

- (1) Biological indexing using a range of indicator plants such as *C. aurantifolia*, *C. macrophylla*, *C. sinensis* or *C. paradisi* (Duncan cultivar) for stem pitting evaluation; and *C. aurantium* or *C. limon* seedlings for seedling yellows evaluation (Roistacher, 1991; Ballester-Olmos *et al.*, 1993).
- (2) Reactivity against the monoclonal antibody MCA13 (Permar *et al.*, 1990), which recognizes an epitope that is well conserved in severe (aggressive) CTV strains but lacking in mild (less aggressive) strains (Pappu, *et al.*, 1993). The reaction with MCA13 is strongly associated with the capacity to induce the decline of trees grafted on sour orange or lemon rootstocks. The majority of CTV strains that produce stem pitting in grapefruit or in sweet orange are MCA13-positive.



## 4.1 Biological indexing

Biological indexing of aggressive CTV strains follows the procedures set out in section 3.3.

## 4.2 Serological tests using MCA13

### 4.2.1 Direct tissue print-ELISA

A complete kit based on the CTV-specific MCA13 monoclonal antibody, including preprinted membranes with positive and negative controls and all reagents, buffers and substrate, is available from Plant Print Diagnostics SL<sup>1</sup>. The method is as follows.

The membranes are tissue printed and blocked as in section 3.5.1. A solution of CTV-specific MCA13 monoclonal antibody linked to alkaline phosphatase (approximately 0.1 µg/ml in PBS) is prepared and poured onto the membranes, covering them, and the membranes are incubated for 3 h at room temperature, with slight agitation. Washing and development of the membranes and reading and interpretation of the results is as in section 3.5.1. The presence of usually small purple-violet precipitates in the vascular region of plant material reveals the presence of a CTV strain of increased aggressiveness.

### 4.2.2 DAS-ELISA

DAS-ELISA is performed according to Garnsey and Cambra (1991) using the method described below. A kit based on the CTV-specific MCA13 monoclonal antibody is available from Plant Print Diagnostics SL<sup>1</sup>.

Coating is performed as described in section 3.5.2. The CTV-specific MCA13 monoclonal antibody linked with alkaline phosphatase is added as conjugate at an appropriate dilution (approximately 0.1 µg/ml in PBS with 0.5% BSA). Incubation, washing, substrate addition and interpretation of results is according to section 3.5.2.

## 5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (Diagnostic protocols for regulated pests).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the virus is found in an area for the first time, the following additional material, if relevant, should be kept in a manner that ensures traceability:

- The original sample should be kept at –80 °C or freeze-dried and kept at room temperature.
- RNA extractions should be kept at –80 °C and/or printed tissue sections and/or spotted plant extracts on paper or nylon membranes should be kept at room temperature.
- RT-PCR amplification products should be kept at –20 °C.

## 6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Centro de Protección Vegetal, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 4.5, 46113 Moncada (Valencia), Spain (Mariano Cambra; e-mail: [mcambra@ivia.es](mailto:mcambra@ivia.es) or [mcambra@mcambra.es](mailto:mcambra@mcambra.es)).

Departamento de Fitossanidade, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Bento Gonçalves 7712, 91540-000 Porto Alegre, Brazil (Edson Bertolini; e-mail: [edson.bertolini@ufrgs.br](mailto:edson.bertolini@ufrgs.br); tel.: +55 (51) 3308 8100).

APHIS-USDA-PPQ-CPHST, 4700 River Road, Riverdale, MD 20737, United States (Laurene Levy; e-mail: [laurene.levy@aphis.usda.gov](mailto:laurene.levy@aphis.usda.gov); tel.: +1 301 851 2078; fax: +1 301 734 8724).

Citrus Research International (CRI), PO Box 28, 1200 Nelspruit, Mpumalanga, South Africa (S.P. Fanie van Vuuren; e-mail: [faniev@cri.co.za](mailto:faniev@cri.co.za)).

Alico, Inc., Suite 100, 10070 Daniels Interstate Court, Fort Myers, FL 33913, United States (Marta Isabel Francis; e-mail: [mfrancis@alicoinc.com](mailto:mfrancis@alicoinc.com); tel.: +1 863 673 4774).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat ([ippc@fao.org](mailto:ippc@fao.org)), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

## 7. Acknowledgements

The first draft of this protocol was written by M. Cambra (IVIA, Spain (see preceding section)), E. Bertolini (IVIA, Spain (see preceding section: current address UFRGS)), L. Levy (APHIS-USDA, United States (see preceding section)), S.P.F. van Vuuren (CRI, South Africa (see preceding section)) and M.I. Francis, Instituto Nacional de Investigación Agropecuaria (INIA) (Uruguay (see preceding section: current address Alico, Inc.)).

Most techniques described were ring tested in the DIAGPRO project financed by the European Union, or evaluated in projects funded by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and the Ministry of Agriculture, Food and Environment, Spain.

## 8. References

The present annex refers to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <https://www.ippc.int/core-activities/standards-setting/ispm>.

**Albertini, D., Vogel, R., Bové, C. & Bové, J.M.** 1988. Transmission and preliminary characterization of *Citrus tristeza virus* strain K. In L.W. Timmer, S.M. Garnsey & L. Navarro, eds. *Proceedings of the 10th Conference of the International Organization of Citrus Virologists (IOCV)*. pp. 17–21. Riverside, CA. ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html)).

**Ballester-Olmos, J.F., Pina, J.A., Carbonell, E., Moreno, P., Hermoso de Mendoza, A., Cambra, M. & Navarro, L.** 1993. Biological diversity of *Citrus tristeza virus* (CTV) isolates in Spain. *Plant Pathology*, 42: 219–229.

**Bar-Joseph, M., Marcus, R. & Lee, R.F.** 1989. The continuous challenge of *Citrus tristeza virus* control. *Annual Review of Phytopathology*, 27: 291–316.

**Bertolini, E., Moreno, A., Capote, N., Olmos, A., De Luis, A., Vidal, E., Pérez-Panadés, J. & Cambra, M.** 2008. Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR. *European Journal of Plant Pathology*, 120: 177–188.

**Broadbent, P., Bevington, K.R. & Coote, B.G.** 1991. Control of stem pitting of grapefruit in Australia by mild strain protection. In R.H. Brlansky, R.F. Lee & L.W. Timmer, eds. *Proceedings of the 11th Conference of the International Organization of Citrus Virologists (IOCV)*. pp. 64–70. Riverside, CA ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html)).

**Cambra, M., Boscia, D., Gil, M., Bertolini, E. & Olmos, A.** 2011. Immunology and immunological assays applied to the detection, diagnosis and control of fruit tree viruses. In A. Hadidi, M. Barba, T. Candresse & W. Jelkmann, eds. *Virus and virus-like disease of pome and stone fruits*, pp. 303–313. St Paul, MN, APS Press. 429 pp.

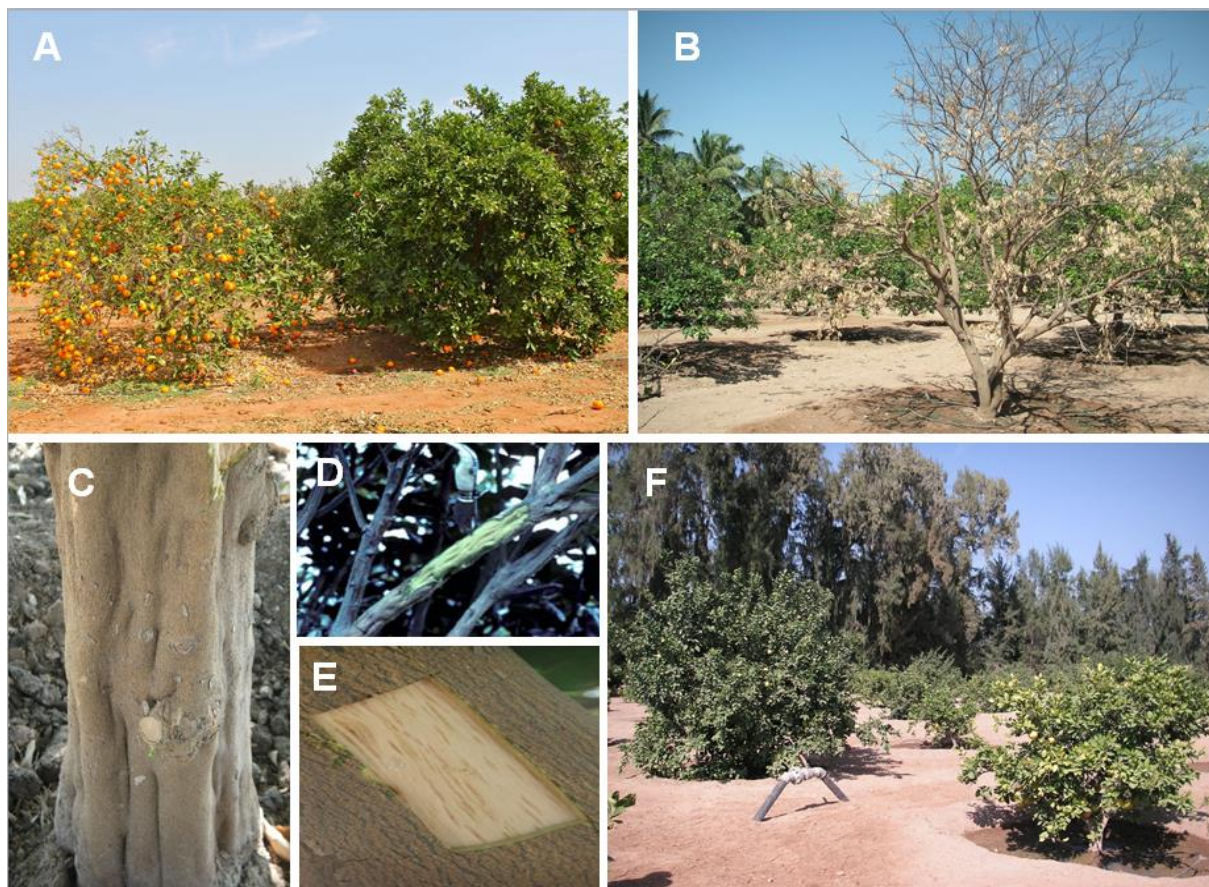
**Cambra, M., Garnsey, S.M., Permar, T.A., Henderson, C.T., Gumph, D. & Vela, C.** 1990. Detection of *Citrus tristeza virus* (CTV) with a mixture of monoclonal antibodies. *Phytopathology*, 80: 103.

- Cambra, M., Gorris, M.T., Marroquín, C., Román, M.P., Olmos, A., Martínez, M.C., Hermoso de Mendoza, A., López, A. & Navarro, L.** 2000a. Incidence and epidemiology of *Citrus tristeza virus* in the Valencian Community of Spain. *Virus Research*, 71: 85–95.
- Cambra, M., Gorris, M.T., Román, M.P., Terrada, E., Garnsey, S.M., Camarasa, E., Olmos, A. & Colomer, M.** 2000b. Routine detection of *Citrus tristeza virus* by direct Immunoprinting-ELISA method using specific monoclonal and recombinant antibodies. In J. da Graça, R.F. Lee & R.K. Yokomi, eds. *Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV)*, pp. 34–41. Riverside, CA. ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html)).
- Cambra, M., Gorris, M.T., Olmos, A., Martínez, M.C., Román, M.P., Bertolini, E., López, A. & Carbonell, E.A.** 2002. European Diagnostic Protocols (DIAGPRO) for *Citrus tristeza virus* in adult trees. In J. da Graça, R. Milne & L.W. Timmer, eds. *Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV)*. pp. 69–77. Riverside, CA, ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html))
- Duran-Vila, N. & Moreno, P.** 2000. *Enfermedades de los cítricos*. Monografías de la Sociedad Española de Fitopatología (SEF) N° 2. Madrid, Ediciones Mundi-Prensa Libros and SEF ([www.sef.es](http://www.sef.es)). 165 pp.
- Garnsey, S.M. & Cambra, M.** 1991. Enzyme-linked immunosorbent assay (ELISA) for citrus pathogens. In C.N. Roistacher, ed. *Graft-transmissible diseases of citrus: Handbook for detection and diagnosis*, pp. 193–216. Rome, FAO. 286 pp.
- Garnsey, S.M. & Lee, R.F.** 1988. Tristeza. In J.O. Whiteside, S.M. Garnsey & L.W. Timmer, eds. *Compendium of citrus diseases*, pp. 48–50. APS Press. St. Paul, MN. 80 pp.
- Garnsey, S.M., Permar, T.A., Cambra, M. & Henderson, C.T.** 1993. Direct tissue blot immunoassay (DTBIA) for detection of *Citrus tristeza virus* (CTV). In P. Moreno, J. da Graça and L.W. Timmer, eds. *Proceedings of the 12th Conference of the International Organization of Citrus Virologists (IOCV)*. pp. 39–50. Riverside, CA. ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html)).
- Gottwald, T.R., Garnsey, S.M., Cambra, M., Moreno, P., Irey, M. & Borbón, J.** 1997. Comparative effects of aphid vector species on increase and spread of *Citrus tristeza virus*. *Fruits*, 52: 397–404.
- Gottwald, T.R., Polek, M.L. & Riley, K.** 2002. History, present incidence, and spatial distribution of *Citrus tristeza virus* in the California Central Valley. In N. Duran-Vila, R. G. Milne & J.V. da Graça, eds. *Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV)*. pp. 83–94. Riverside, CA, ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html)).
- da Graça, J.V. & van Vuuren, S.P.** 2010. Managing *Citrus tristeza virus* losses using cross protection. In A.V. Karasev & M.E. Hilf, eds. *Citrus tristeza virus complex and tristeza diseases*, pp. 247–260. Eagan, MN, APS Press. 304 pp.
- Harju, V.A., Henry, C.M., Cambra, M., Janse, J. & Jeffries, C.** 2000. Diagnostic protocols for organisms harmful to plants-DIAGPRO. *EPPO Bulletin*, 30: 365–366.
- Harper, S.J.** 2013. *Citrus tristeza virus*: Evolution of complex and varied genotypic groups. *Frontiers in Microbiology*, doi:10.3389/fmicb.2013.00093.
- Harper, S.J., Dawson, T.E. & Pearson, M.N.** 2008. Molecular analysis of the coat protein and minor coat protein genes of New Zealand *Citrus tristeza virus* isolates that overcome the resistance of *Poncirus trifoliata* (L.) Raf. *Australian Plant Pathology*, 37: 379–386.
- Ilharco, F.A., Sousa-Silva, C.R. & Alvarez-Alvarez, A.** 2005. First report on *Toxoptera citricidus* (Kirkaldy) in Spain and continental Portugal. *Agronomia Lusitana*, 51: 19–21.
- Licciardello, G., Raspagliesi, D., Bar-Joseph, M. & Catara, A.** 2012. Characterization of isolates of *Citrus tristeza virus* by sequential analyses of enzyme immunoassays and capillary electrophoresis-single-strand conformation polymorphisms. *Journal of Virological Methods*, 181: 139–147.
- Marroquín, C., Olmos, A., Gorris, M.T., Bertolini, E., Martínez, M.C., Carbonell, E.A., Hermoso de Mendoza, A.H. & Cambra, M.** 2004. Estimation of the number of aphids carrying *Citrus tristeza virus* that visit adult citrus trees. *Virus Research*, 100: 101–108.

- Moreno, P., Ambros, S., Albiach-Martí, M.R., Guerri, J. & Peña, L.** 2008. *Citrus tristeza virus*: A pathogen that changed the course of the citrus industry. *Molecular Plant Pathology*, doi:10.1111/J.1364-3703.2007.00455.X.
- Nikolaeva, O.V., Karasev, A.V., Powell, C.A., Gumpf, D.J., Garnsey, S.M. & Lee, R.F.** 1996. Mapping of epitopes for *Citrus tristeza virus*-specific monoclonal antibodies using bacterially expressed coat protein fragments. *Phytopathology*, 86: 974–979.
- Olmos, A., Cambra, M., Esteban, O., Gorris, M.T. & Terrada, E.** 1999. New device and method for capture, reverse transcription and nested PCR in a single closed tube. *Nucleic Acids Research*, 27: 1564–1565.
- Pappu, H.R., Manjunath, K.L., Lee, R.F. & Niblett, C.L.** 1993. Molecular characterization of a structural epitope that is largely conserved among severe isolates of a plant virus. *Proceedings of the National Academy of Sciences of the United States of America*, 90: 3641–3644.
- Permar, T.A., Garnsey, S.M., Gumpf, D.J. & Lee, R.** 1990. A monoclonal antibody that discriminates strains of *Citrus tristeza virus*. *Phytopathology*, 80: 224–228.
- Roistacher, C.N.** 1991. *Graft-transmissible diseases of citrus: Handbook for detection and diagnosis*. Rome, FAO. 286 pp.
- Román, M.P., Cambra, M., Juárez, J., Moreno, P., Durán-Vila, N., Tanaka, F.A.O., Alves, E., Kitajima, E.W., Yamamoto, P.T., Bassanezi, R.B., Teixeira, D.C., Jesús Jr, W.C., Ayres, J.A., Gimenes-Fernandes, N., Rabenstein, F., Girotto, L.F. & Bové, J.M.** 2004. Sudden death of citrus in Brazil: A graft transmissible, bud union disease. *Plant Disease*, 88: 453–467.
- Ruiz-García, N., Mora-Aguilera, G., Rivas-Valencia, P., Ochoa-Martínez, D., Góngora-Canul, C., Loeza-Kuk, E.M., Gutiérrez-E, A., Ramírez-Valverde, G. & Álvarez-Ramos, R.** 2005. Probability model of *Citrus tristeza virus* detection in the tree canopy and reliability and efficiency of direct immunoprinting-ELISA. In M.E. Hilf, N. Duran-Vila & M. A. Rocha-Peña, eds. *Proceedings of the 16th Conference of the International Organization of Citrus Virologists (IOCV)*. pp. 196–204. Riverside, CA. ([www.iocv.org/proceedings.html](http://www.iocv.org/proceedings.html)).
- Sackett, D.L., Haynes, R.B., Guyatt, G.H. & Tugwell, P.** 1991. *Clinical epidemiology: A basic science for clinical medicine*, 2nd edn. Boston, MA, Little Brown and Co. 441 pp.
- Saponari, M., Manjunath, K. & Yokomi, R.K.** 2008. Quantitative detection of *Citrus tristeza virus* in citrus and aphids by real-time reverse transcription-PCR (TaqMan). *Journal of Virological Methods*, 147: 43–53.
- Terrada, E., Kerschbaumer, R.J., Giunta, G., Galeffi, P., Himmler, G. & Cambra, M.** 2000. Fully “Recombinant enzyme-linked immunosorbent assays” using genetically engineered single-chain antibody fusion proteins for detection of *Citrus tristeza virus*. *Phytopathology*, 90: 1337–1344.
- Timmer, L.W., Garnsey, S.M. & Graham, J.H.** 2000. *Compendium of citrus diseases*. St Paul, MN, APS Press. 92 pp.
- Vela, C., Cambra, M., Cortés, E., Moreno, P., Miguët, J., Pérez de San Román, C. & Sanz, A.** 1986. Production and characterization of monoclonal antibodies specific for *Citrus tristeza virus* and their use for diagnosis. *Journal of General Virology*, 67: 91–96.
- Vidal, E., Yokomi, R.K., Moreno, A., Bertolini, E. & Cambra, M.** 2012. Calculation of diagnostic parameters of advanced serological and molecular tissue-print methods for detection of *Citrus tristeza virus*. A model for other plant pathogens. *Phytopathology*, 102: 611–619.
- Yokomi, R.K., Garnsey, S.M., Civerolo, E.L. & Gumpf, D.** 1989. Transmission of exotic citrus tristeza isolates by a Florida colony of *Aphis gossypii*. *Plant Disease*, 73: 552–556.
- Yokomi, R.K., Saponari, M. & Sieburth, P.J.** 2010. Rapid differentiation and identification of potential severe strains of *Citrus tristeza virus* by real-time reverse transcription-polymerase chain reaction assays. *Phytopathology*, 100: 319–327.
- Zebzami, M., Garnsey, S.M., Nadori, E.B. & Hill, J.H.** 1999. Biological and serological characterization of *Citrus tristeza virus* (CTV) isolates from Morocco. *Phytopathologia Mediterranea*, 38: 95–100.

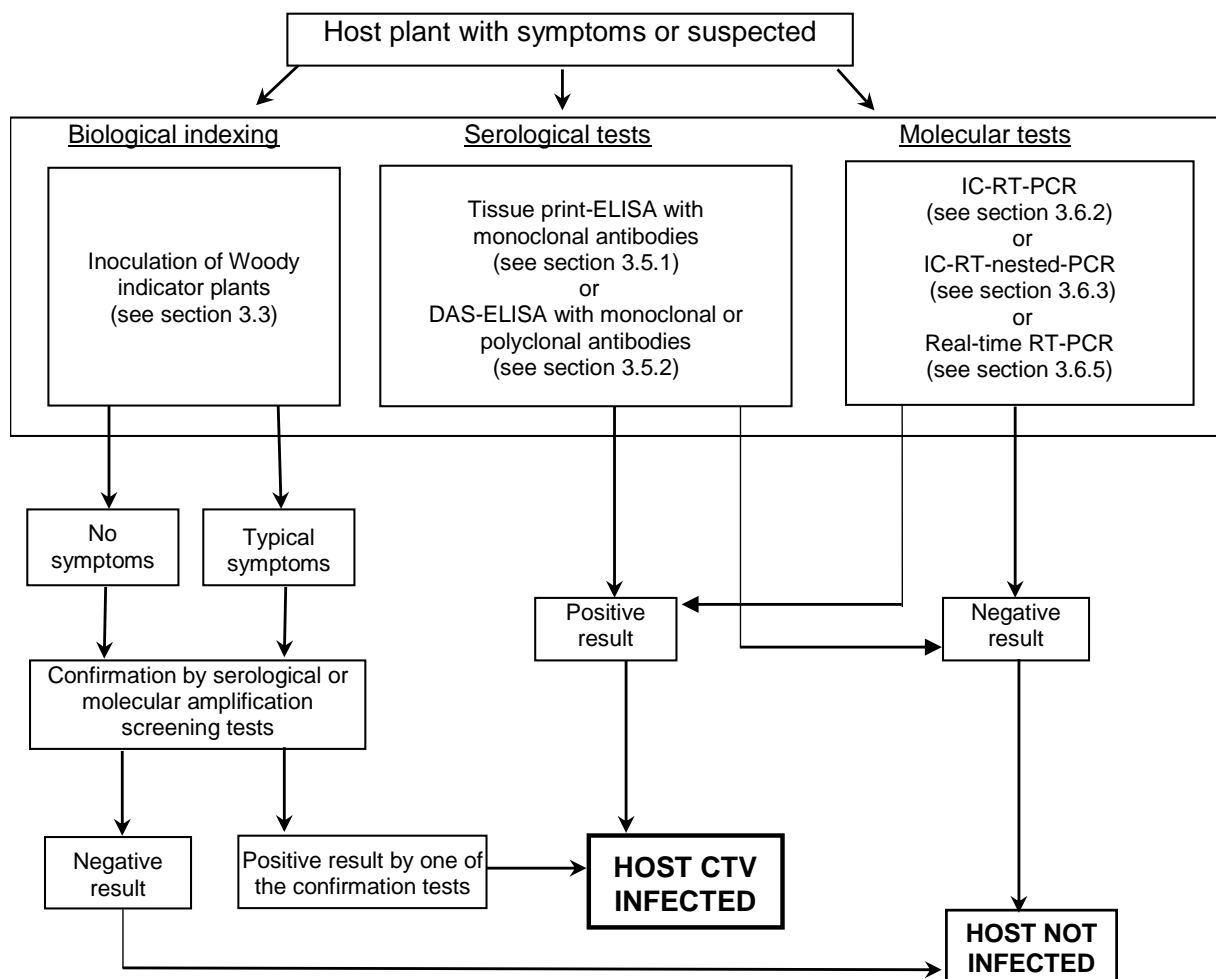


## 9. Figures



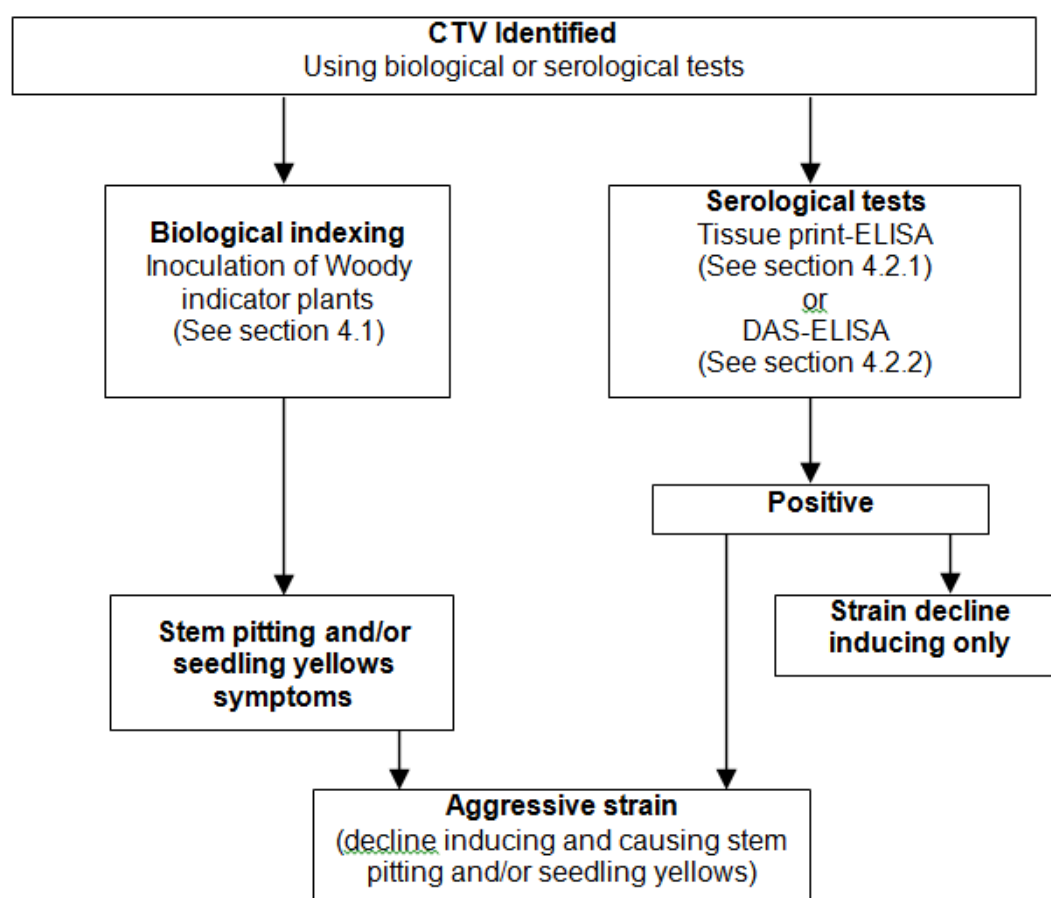
**Figure 1.** Symptoms of *Citrus tristeza virus* (CTV) infection: (A) tristeza syndrome or decline of sweet orange grafted on sour orange infected with CTV (left) and symptomless tree (right); (B) collapse or quick decline of grapefruit grafted on sour orange; (C) stem pitting on the trunk of grapefruit grafted on Troyer citrange caused by an aggressive CTV strain; (D) severe stem pitting on the branches of grapefruit; (E) stem pitting on the trunk of sweet orange grafted on Cleopatra mandarin; and (F) pronounced stunting of CTV-infected sweet orange trees grafted on Carrizo citrange (right) compared with a healthy tree (left).

Photo courtesy (A) P. Moreno; (B, C, E) M. Cambra; (D) L. Navarro; and (F) M. Cambra and J.A. Pina. All from Instituto Valenciano de Investigaciones Agrarias, IVIA, Moncada, Spain.



**Figure 2.** Flow chart for the detection and identification of *Citrus tristeza virus* (CTV).

DAS, double antibody sandwich; ELISA, enzyme-linked immunosorbent assay; IC, immunocapture; PCR, polymerase chain reaction; RT, reverse transcription.



**Figure 3.** Flow chart for the identification of aggressive strains of *Citrus tristeza virus* (CTV). DAS, double antibody sandwich; ELISA, enzyme-linked immunosorbent assay.

#### Publication history

*This is not an official part of the standard.*

2004-11 SC introduced original subject: *Citrus tristeza virus* (2004-021).

2006-04 CPM-1 added subject under work programme topic: Viruses and phytoplasmas (2006-009).

2006-04 CPM-1 (2006) added work programme topic: Nematodes (2006-008).

2014-04 Expert consultation.

201-01 SC approved for member consultation (2015\_eSC\_May\_02).

2015-02 Member consultation.

2015-12 DP drafting group reviewing draft DP and responses to member comments.

2015-11 Submitted to SC for approved for DP notification period (2016\_eTPDP\_Feb\_02).

2016-03 SC e-decision for approval to be submitted to the 45 day DP notification period (2016\_eSC\_May\_10).

2016-08 SC adopted DP on behalf of CPM (with no objections received).

**ISPM 27. Annex 15.** *Citrus tristeza virus* (2016). Rome, IPPC, FAO.

2017-01 IPPC Secretariat made minor editorial changes (English version only).

Publication history last updated: 2017-01.

## IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

### Organization

- ◆ There are over 180 contracting parties to the IPPC.
- ◆ Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- ◆ Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- ◆ IPPC liaises with relevant international organizations to help build regional and national capacities.
- ◆ The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).



### International Plant Protection Convention (IPPC)

Viale delle Terme di Caracalla, 00153 Rome, Italy  
Tel: +39 06 5705 4812 - Fax: +39 06 5705 4819  
Email: [ippc@fao.org](mailto:ippc@fao.org) - Web: [www.ippc.int](http://www.ippc.int)