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## **NAPPO DIAGNOSTIC PROTOCOL**

### **DP No. 01 Citrus Tristeza Closterovirus (CTV)**

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## **Protocol: Citrus Tristeza Closterovirus (CTV)**

### **1. Pest information**

Citrus tristeza closterovirus (CTV) is the cause of several economically serious diseases of citrus. CTV probably originated in Asia and was disseminated to most citrus producing countries by infected propagative material. Subsequent spread by aphid vectors caused epidemics in the 20th century. Millions of trees planted on sour rootstocks have been lost to tristeza decline in many countries, including the United States, Spain, and Brazil. Trees on tolerant or resistant rootstocks may be affected by stem pitting strains that can reduce yields and quality (Bar-Joseph *et al.*, 1989; Garnsey *et al.*, 1998; Lee and Bar-Joseph, 2000).

CTV probably occurs in all citrus growing areas worldwide, although its incidence varies. CTV is usually detected in isolated citrus trees and still is not considered epidemic in many countries such as Albania, Algeria, Cyprus, Egypt, France, Greece, Italy, Jordan, Lebanon, Libya, Morocco, Palestine, Portugal, Syria, Tunisia and Turkey and in some Central and South America countries such as Belize, Chile, Ecuador, El Salvador, Honduras, Mexico and Nicaragua. CTV is considered to cause the most damaging viral disease of citrus. In Argentina, with its occurrence in 1945, it caused the death of approximately 10 million trees, and in 1959 this number increased to 18 million (Rocha-Peña *et al.*, 1995). Brazil reported over 10 million of trees lost in 1958. In the sixties in Spain and in the eighties in Venezuela, CTV destroyed approximately 16 million of trees (Rocha-Peña *et al.*, 1995).

In Mexico, CTV was first detected in 1983 in the State of Tamaulipas, and in 1986 in Veracruz; both outbreaks were quickly eradicated. The virus was subsequently reported in numerous other Mexican States.

Since 1939, the disease has also been observed in California, United States. Observations made by Halma, Smoyer and Schwalm (1944, 1945) indicated that quick decline was similar to the "tristeza" disease reported in Brazil. Fawcett and Wallace (1946) at the same time as Meneghini (1946), reported that tristeza in Brazil was caused by a virus. It can be concluded that CTV caused the death of at least 3 million citrus trees in California. Grant and Schneider (1951) reported for the first time the occurrence of CTV in Florida, although no doubt its presence in the state goes back many years before its identification. The tristeza strains identified in the state of Florida are different than those present in California. In Texas, Olson and Sleeth (1954) and Olson and McDonald (1954), found 'Meyer' lemon trees infected with tristeza. The 'Meyer' lemon was the first host reported in the State of Arizona (Carpenter, 1956).

#### **1.1 Symptoms**

Different isolates of CTV vary greatly in the symptoms they produce in different citrus hosts. Some are extremely mild and cause little injury, even in sensitive hosts. Some are severe in specific hosts and benign in others, and some can cause very severe reactions in a wide range of hosts. Different isolates can have a wide range of severity in the same hosts and, conversely, the same isolate can vary in symptoms expressed in different hosts. Graft inoculation of a range of different indicator plants is commonly used to develop a profile of the biological properties of CTV and to predict probable severity in commercial hosts (Garnsey *et al.*, 2005). Common CTV symptoms include vein clearing, stunting, yellowing, several types of stem pitting, and seedling yellows (SY) (Moreno *et al.*, 2008).

CTV isolates and symptom phenologies can be categorized as follows: i) tristeza decline or quick decline strains which induce a collapse of citrus grown on sour orange rootstock; ii) mild strains that are symptomless in citrus grown on CTV-tolerant or resistant rootstocks; iii) seedling yellows strains which produce stunting and chlorosis in seedlings of sour orange, grapefruit, or Eureka lemon in a greenhouse virus index (generally considered to indicate severe strains); iv) stem pitting strains which induce moderate to severe stem pitting in bark and adjacent wood in branches and trunks of sweet orange, grapefruit, or mandarins regardless of rootstock.

There are essentially two commercially important CTV diseases of citrus. One is the classic tristeza decline that is associated with a decline of trees grafted on sour orange rootstocks caused by a virus-induced phloem necrosis at the budunion (Figures 1a, 1b). Tristeza decline has caused severe losses in many citrus growing areas, including California, and still threatens citrus industries having sour orange as the main rootstock. The CTV-induced decline can be avoided by use of decline-tolerant or –resistant rootstocks in lieu of sour orange; however the use of these rootstocks may result in other diseases and horticultural problems (Moreno *et al.*, 2008).

The second major CTV-induced disease is stem pitting. In contrast to decline, stem pitting can reduce the vigor and production of infected scions regardless of rootstock. Some rootstocks can be affected by stem pitting even when the scion is not. CTV stem pitting has caused serious losses in many other citrus growing areas (Moreno and Garnsey, 2005; Rocha-Peña *et al.*, 1995). One option for control of CTV-stem pitting is to avoid cultivars that are highly sensitive and grow only stem pitting tolerant mandarins on tolerant rootstocks. Another option is mild strain cross protection (Moreno *et al.*, 2008). This has been used in several countries for grapefruit and sweet orange, but cross protection is confined to closely related isolates within a genotype and may also be cultivar specific. Protecting isolates alone may also adversely affect production (Moreno *et al.*, 2008).

## 1.2 Transmission

CTV can be transmitted by grafting and semi-persistently by one of several different species of aphid including *Toxoptera citricida* (Kirkaldy), *T. aurantii* (Boyer de Fonscolombe), *Aphis gossypii* (Glover), and *A. spiraecola* (Patch) (Moreno *et al.*, 2008). Efficiency of aphid transmission can be affected by the donor and receptor host, by the virus isolate and its ability to replicate in a given host, by the vector species and its biology, and by environmental and horticultural factors. The most efficient aphid vector is *T. citricida* (the Brown Citrus Aphid). However, aphid transmission by other species is efficient enough to cause economically significant spread of CTV (Rocha-Peña *et al.*, 1995). *T. citricida* was detected in February 2000 in the northern part of the states of Quintana Roo and Yucatán, México. It has now been detected in the states of Yucatán, Quintana Roo, Oaxaca, Puebla, and Campeche in México. In the United States, *T. citricida* has been reported from Florida but not from California, Arizona, or Texas. Extensive research has been done on the epidemiology of CTV in different citrus growing areas and how it is influenced by aphid, host and virus isolate factors (Gottwald *et al.*, 1999). Techniques have been developed and tested for monitoring and predicting rates and patterns of spread (Hughes *et al.*, 2001). In general, once natural infection rates reach 0.1% to 0.5%, infection rates increase rapidly and suppression by tree removal becomes difficult. In areas with active natural spread, the level of actual infection will exceed that determined by testing since some recent infection will not be detected. CTV has not been demonstrated to be seed-transmitted. Most species of Citrus and specific related genera of Rutaceae (Aurantioideae sub-family) have been

reported to be hosts of CTV. However, trifoliates (*Poncirus trifoliata*) and many of their hybrids are resistant to CTV.

## **2. Taxonomic information**

CTV is a member of the closterovirus group. The pathogen itself is a flexible virion of about 2,000 X 11 nm and has a non-segmented, positive-sense, single stranded Ribonucleic Acid (RNA) genome of approximately 20 Kb. Studies on the molecular properties of CTV have revealed that it is a complex virus group. Three major genotype groups have been recognized along with some variations within these. Recombinants between different genotypes and the fact that single “pure” isolates are actually populations of RNA variants further increase the complexity (Rubio *et al.*, 2001). Associations have been established between biological reactions and various genetic markers, but at this point no specific pathogenicity determinants for decline or stem pitting have been positively identified (Nikolaeva *et al.*, 1998, Hilf and Garnsey 2000, Moreno *et al.*, 2008).

## **3. Diagnostic**

CTV can be detected by biological indexing and various non-biological methods including light and electron microscopy, serology and a variety of molecular-based techniques. The latter include several types of reverse transcription polymerase chain reaction (RT-PCR), including an immunocapture PCR with multiple molecular markers (MMM) (Hilf *et al.*, 2005) and real time PCR (Ruiz-Ruiz *et al.*, 2009), SSCP analysis (Rubio *et al.*, 1996), oligoprobes (Narvaez *et al.*, 2000) and RFLP analysis (Gillings *et al.*, 1993). The following techniques are approved by NAPPO: biological indexing, ELISA, and immunoprint ELISA.

It is important to consider that sampling to detect CTV infected propagative material must be done during the citrus flushing season, coinciding with less hot months (when the monthly average temperature is around 27 °C and with little fluctuation during the year). When moving plants within Mexico, sampling is done regardless of temperature. Each sample is made up of 10-15 cm shoots, of 5 trees, taken from the four tree quadrants. If plants are too small and have no shoots, petioles can be used.

In California, where temperatures vary more than in Mexico, CTV incidence is very low during the winter and summer months (when temperatures are extreme) in most of the regions of California. The ideal season to detect CTV is between April and June or in October/November. This allows testing to be performed when incidence is high, providing more reliable diagnostics (Dodds *et al.*, 1987).

### **3.1 ELISA (Enzyme Linked Inmunosorbent Assay)**

To detect CTV, the Enzyme Linked Inmunosorbent Assay (ELISA) serological technique is used. This technique is based in the capability of certain proteins, known as antibodies, to recognize and link a specific antigen associated to the pathogen. This is a reliable and quick technique to detect plant pathogen virus. Some variants have been developed, but so far the DAS-ELISA (Double Antibody Sandwich) direct method is the most commonly used (Salazar, 1990). This technique consist of specific antibodies absorption to a polystyrene plate which later on the antigen is added to and then reacts to the antibodies adhered to the plate. Then, an enzymatic conjugate is added (second antibody) to form the complex antibody-antigen-antibody.

The specificity of monoclonal antibodies and the ease to trade them (since they are homogenous and stable), in addition to the advantages offered by the technique such

as sensibility, low cost, speed, reliability and capability for mass usage, have favoured the application of the ELISA technique in all citrus growing countries to detect CTV, using monoclonal antibodies capable to recognize any virus isolate without any reaction with components of the host plant.

### 3.1.1. Procedure

The antibodies developed by Nikolaeva *et al.*, (1995) and/or Lee (primary antibodies CREC 1052, CREC 27, CREC 28, CREC 29, CREC 30, or CREC 31 and the secondary antibody G604) are used in California. Agdia and BioReba commercial kits for CTV are presently used in Mexico. The United States uses two different techniques, one by the Citrus Clonal Protection Program (CCPP) using Gumpf antibodies and the other used by the Germplasm Repository (NCGRCD) which uses Lee antibodies (Lee *et al.*, 2005).

#### 3.1.1.1 Procedure of the Citrus Clonal Protection Program (CCPP) (Clark and Adams, 1977, modified for tristeza by J.M. Díaz of CCPP).

##### A. Sample preparation

1. Collect young branches from the four tree quadrants (or eight quadrants for trees at risk of field infection).
2. Remove the bark from the branch, calculate approximately 1.0 g and cut in pieces of 1-2 mm.
3. Prepare the samples to 1:10 (p/v) in extraction buffer, using the KLECO tissue pulverizer or the Ultra-Turaz T25 Tissumizer<sup>1</sup>.

<b>Buffer PBS</b>	
NaCl	8.0 g
KH <sub>2</sub> PO <sub>4</sub>	0.2 g
Na <sub>2</sub> HPO <sub>4</sub> .7H <sub>2</sub> O	2.17 g
KCl	0.2 g
NaN <sub>3</sub>	0.2 g
Adjustd the pH to 7.4, 1 L of H <sub>2</sub> O.	

PBSTween Buffer: PBS Buffer + Tween20 2 ml/L

Extraction Buffer: Buffer PBSTween + 20 g/L PVP40 polyvinylpyrrolidone (Sigma)

4. Samples can be frozen and thaw before preparing the plate. This helps lower the bottom of the optical density (recommended but not required).

<sup>1</sup> Manufacturing: Kleco Tissue Pulverizer, 14097 Ave 272, Visalia, CA 93292, 559-732-3785 or Tissuemizer, Ultra Turraz T25, Janke & Kunkel

## B. Preparation of ELISA<sup>2</sup> plates

1. Place 200 µl of  $\gamma$ -globulin in buffer coat in each well of the plate. Incubate for 4 hours at 37 °C. Normal concentration is 1-2 µg/ml. Ideal concentration must be determined by limiting dilution.

<b>Buffer coat</b>	
Na <sub>2</sub> CO <sub>3</sub>	1.59 g
NaHCO <sub>3</sub>	2.93 g
NaN <sub>3</sub>	0.2 g
Adjust the pH at 9,6, in 1 L of H <sub>2</sub> O.	

2. Wash the plate with PBS-Tween. Let it settle for 3 minutes. Repeat washing 3 times emptying the plate each time.

## C. Sample addition

1. Place proportionally 200 µl of samples in each of two wells. Incubate at 6 °C overnight or at 37 °C for 4 hours.
2. Wash the plate 3 times as in step B.2. and wash the plate 3 times with distilled water, which helps lower the bottom of optical density.

## D. Addition of secondary and conjugated antibodies

1. Place proportionally 200 µl of secondary antibody [according to Drs. A and O Karasev (Nikolaeva *et al.*, 1995)] in each well to the ideal pre-determined dilution. Incubate at 37 °C for 4 hours. For this step, use the conjugated buffer.  
Enzyme conjugate buffer: Buffer PBSTween + 20 g/L PVP40 polivinylpirrolidona + 2 g/L ovalbumin (Sigma A-7030)
2. Wash the plate 3 times as in step B.2.
3. Place proportionally 200 µL of commercial conjugate (Sigma A-8025 Anti-Rabbit Igl Alkaline Phosphatase Antibody, develop on goat), which usually has a dilution of 1:8.000 to 1:10.000. Incubate at 37 °C for 4 hours.

## E. Substratum addition (P-nitrophenyl phosphate disodium, Sigma S-0942, 5 mg tablets)

1. Wash the plate 3 times as indicated in step B.2.
2. Place proportionally 200 µl of recently prepared substrate at a concentration of 0,6 -- 1,0 mg/ml of p-nitrophenyl phosphate in substrate buffer.

<b>Substrate Buffer</b>	
Diethanolamine	97.0 ml
NaN <sub>3</sub>	0.2 g
Adjust the pH to 9.8, in 1 L of H <sub>2</sub> O.	

## F. Evaluation of the plate

1. Incubate at room temperature until you can see the reaction. Reaction time is usually ~ 30 minutes.
2. Evaluate the results to measure absorbency at 405 nm in the plate reader<sup>3</sup> . Positive samples are considered those that are two times greater than the average value of the negative control (Clark, 1977).

<sup>2</sup> Microtiter plates: Immulon IV Flat plates, Fisher #14245153 (50 plates/pack)

<sup>3</sup> ELISA plate reader: Molecular Devices EMax

### 3.1.1.2 Procedure of the Germplasm Repository (NCGRCD)

#### A. Selection and sample preparation

##### A1. Bark samples

1. Collect twigs that have just matured (fresh and dark green) from four sides of the tree. Bark should be easily stripped from this type of tissue.
2. Place the flushes from each tree in a labeled plastic bag. Store the samples in an ice chest if it will be more than a few minutes before the samples are processed.
3. In the laboratory, strip the bark from each of the collected flushes.
4. Estimate about 0.25 g of bark, and chop into 1 – 2 mm long pieces.
5. Add to 5 ml of coating buffer in a 50 ml test tube.
6. Homogenize for 15 seconds using a tissue miser or Polytron type homogenizer.
7. Store at 4 °C overnight before loading into the ELISA plate for maximum extraction.

##### Alternative method:

1. Estimate about 0,20 g of bark and chop into 1-2 mm long pieces.
2. Place in a 2 ml centrifuge tube with screw cap, add 2 bbs and fill the tube (1.5 ml) with coating buffer.
3. Homogenize for 5 minutes using a mini-bead beater.
4. Store at 4 °C overnight before loading into the ELISA plate for maximum extraction.

Chopped midribs or leaf petioles can also be used in these procedures.

Coating Buffer	
Na <sub>2</sub> CO <sub>3</sub>	1.59 g
NaHCO <sub>3</sub>	2.93 g
adjust to pH 9.6 with HCl in 1 L H <sub>2</sub> O.	

##### A2. Leave samples

1. Collect two leaves from each of four sides of the tree. They must be fully expanded leaves from the last growth flush. In the field, these can be stapled together and labelled with a magic marker on the underside of the last leaf.
2. Roll one leaf from each side of the tree using a leaf roller and collect the sap into a 1.5 ml microcentrifuge tube. Keep the other four leaves as a backup sample.
3. Centrifuge for 5 minutes.
4. Pipet 200 µl of the supernatant into a dilution tube filled with 800 µl of coating buffer. Mix by pipetting the solution up and down a few times.

##### Direct extraction using stem from new, tender flush tissue

1. After step 4 (wash procedure), fill all sample wells of the plate proportionally with 200 µl Aliquots of coating buffer containing 0.1 % Tween-20.
2. Chop the stem in 1-2 mm long pieces using pruning shears.
3. Drop the pieces of tissue directly into the wells filled with the buffer.
4. Incubate overnight at 4 °C.

#### B. Preparation of control

For BSD-ELISA usage of glycerol-prepared controls stored at –20°C is preferred for standardization purposes (Lee *et al.*, 2005). A healthy (CTV -) and positive (CTV +) control is included with the detection kit. These controls are ready to load into the ELISA plate. 100 ul aliquots are loaded into wells that are pre-filled with 100 ul of coating buffer with 0.2% Tween 20.

Dried tissue can be used for the controls. Use about 50 mg of tissue in 5 ml of coating buffer. Let the dried tissue hydrate as long as possible up to 24 hours before homogenizing. A good strategy is to hydrate the controls early in the morning, grind late in the afternoon, and extract overnight at 4°C with the candidate samples.

C. Preparation of ELISA plates

1. Coat the ELISA plates with polyclonal antiserum made against purified CTV (preparation of IgG CREC27, CREC28, CREC31, CREC1051, or CREC 1052). Dilute the “CREC29 coating buffer” with the coating buffer to the concentration indicated on the coating buffer tube (usually 1:5,000). Add 200 µl of the diluted antiserum to each of the wells on the plate except the uncoated control well; we usually leave two wells as an uncoated control, usually wells A11 & A12. Incubate 1 to 3 hours at room temperature, or overnight at 4 °C. (Note that when using overnight incubations, higher dilutions can be used for the coating antiserum. For example, if running the step at room temperature using a 1/5,000 dilution, then for overnight incubation in the cold, use a 1/10,000 dilution.)

D. Wash

1. Shake the solution from the plate and wash 3X under a stream of deionized water.
2. Fill the plate with PBST, and let set for 5 minutes or longer (let set for at least 30 minutes on the first wash).

<b>PBST</b>	
NaCl	8.0 g
KH <sub>2</sub> PO <sub>4</sub>	0.2 g
Na <sub>2</sub> HPO <sub>4</sub>	1.15 g (anhydrous)
KCl	0.2 g
Adjust to pH 7,4 using HCl , 1 L.	
Add 1 ml of Tween 20	

3. Repeat steps 1 and 2 twice.
4. Repeat step 1.
5. Invert and tap on a clean paper towel to drain excess water immediately before the next step. Make sure the plate does not dry out.

E. Samples

1. Fill all wells of the ELISA plate with 100 µl of coating buffer containing 0.2% Tween-20.
2. Add 100 µl aliquot of the sample per well, use two wells per sample. Mix the samples with the buffer already in the well by pipetting up and down in the well a few times.
3. Incubate overnight at 4 °C.
4. Wash the plate following the wash procedure.

F. Secondary antibody

1. Dilute the G-604 intermediate buffer to a 1:20,000 dilution in conjugate buffer <sup>4</sup>.

<b>Conjugate buffer (500 ml)</b>	
PBST	500 ml
PVP-40	10.0 g
(Bovine serum albumin, Fraction V).	1.0 g

2. Add 100 µl aliquots of this solution to all wells in the plate.
3. Incubate for 1 hour at 37 °C, or overnight at 4 °C.
4. Wash the plate following the wash procedure.

G. Conjugate

1. Dilute Rabbit Anti-goat conjugate with alkaline phosphatase to the recommended dilution on the tube (usually 1:30.000) in conjugate buffer.
2. Add 100 µl aliquots of this solution to all wells in the plate.
3. Incubate for 3 hours at 37 °C or overnight at 4°C.
4. Wash the plate following the wash procedure.

H. Reaction

1. Add 0.6 mg/ml of substrate (4-nitrophenyl phosphate hexahydrate) to coating buffer. Make sure the coating buffer is fresh, unless it was prepared using NaN<sub>3</sub> or it is not older than a few days and stored at 4°C.
2. Add 200 µl aliquots of this solution to each well.
3. Incubate at room temperature.
4. Color usually develops very slowly. Read the plate after one or two hours, and on the next morning. Set the plate reader to blank on the uncoated wells. This will subtract the absorption of light caused by the plate and the buffer. No significant background reaction should develop during an overnight development of the plate. If this is the case, the secondary antibody should be diluted further.

I. Evaluation

1. Evaluate the controls first. The uncoated control should not give any reaction at all. This will not be visible on the plate readings, since the reader blanks on these wells, but can be seen directly on the plate. Reaction in the uncoated wells indicates improper washing of the plates after the conjugate step, improper preparation of one of the buffers, or loss of the specificity of the antibodies against the antigen.
2. It is not unusual for the buffer only wells to show some reaction, sometimes even more than that of the healthy control.
3. The healthy control wells should show none or very low reaction.
4. A good plate will show OD405 values under 0.050 for the healthy and buffer only wells, and values over 1.000 for the positive controls. In that case, a sample is considered positive if the average OD value is more than 0.100. Antibody dilutions need to be adjusted when higher values are obtained for the negative controls, or lower values obtained for the positive controls.

Note: For all protocols, the final reaction volume in each step is the same. For this case, it is 100 µl.

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<sup>4</sup> Each lot of G-604 must be calibrated making a series of dilutions.

### 3.1.1.3 Procedure used with the commercial Agdia kit

1. Preparation of the sample
  - a. Remove the bark from each shoot<sup>5</sup> of the sample collected. Weigh 0,2 g of bark and cut into pieces of 1-2 mm.
  - b. Empty the tissue in a 50 ml tube and add 2 ml of extraction buffer.

<b>Extraction buffer solution (Macerate of plant samples)</b>	
Sodium sulfite (anhydrous)	1.3 g
Polyvinylpyrrolidone (PVP) Molecular weight of 24 - 40,000	20.0 g
Sodium Azide	0.2 g
Ground Egg (chicken) albumin, Grade II	2.0 g
Tween-20	20.0 g
Dissolve in 1Lt of Phosphate buffer solution 1X (PBST)	

Note: Adjust the pH to 7,4 (+/- 0,2). Store at 4 °C on refrigeration

- c. Blend for 15 seconds using a tissue homogenizer.
  - d. For better extraction, store at 4 °C overnight before placing the samples on the ELISA plate.
2. Preparation of ELISA plates
  - a. Place 100 µl of the specific antibody dilution for CTV (Dilution 1:200<sup>6</sup>) in each well plus the coating buffer solution taking into account the sample distribution pattern, considering the samples and negative and positive controls with their replicates. Incubate in wet chamber for 2 hours at 37 +/- 2 °C or overnight at 4 °C.

<b>Coating Buffer</b>	
Sodium carbonate (anhydrous)	1.59 g
Sodium bicarbonate	2.93 g
Sodium azide	0.2 g
Adjust the pH at 9,6 (+/- 0,2). Store at 4 °C	
Dissolve in 1 L of distilled water	

3. Plate washing
  - a. Wash the plate 3 to 5 times<sup>7</sup> with PSTB-T 1X

<b>Phosphate (PBST) buffer solution (wash)</b>	
Sodium chloride	8.0 g
Dibasic sodium phosphate (anhydrous)	1.15 g
Monobasic potassium phosphate (anhydrous)	0.2 g
Potassium chloride	0.2 g
Tween 20	0.5 g/ml
Dissolve in 1L of distilled water	

<sup>5</sup> Leaves main veins or petioles can also be used.

<sup>6</sup> Dilution commonly used. Follow the manufacturer's instructions.

<sup>7</sup> Discard the plate contents in a sink, shake hard several times on paper towel. Fill each of the sensitized wells with the wash buffer, starting on the first line, then the second line and so on, to cover all the needed wells, let stand for 1 minute and discard again the plate contents, shake well over the paper towel.

Adjust the pH to 7,4 (+/- 0,2), washing solution can be prepared as a concentrate first and then diluted. Prepare a concentration of 20X (20 times concentrated).

4. Sample addition
  - a. Plant samples of interest are crushed or homogenized with extraction grinding buffer. Add 100  $\mu$ l of the homogenized tissue to each well, with its replicates, in the previously selected wells.
5. Control preparation
  - a. In each of the wells associated with the negative controls, place either a 100  $\mu$ l of the extraction solution by itself or commercial negative control. It is recommended not to use the wells close to the edges since they can develop non-specific reactions (Salazar, 1990). Dilute the positive control and place 100  $\mu$ l of this in the wells. Incubate in wet chamber for 2 hours at 37 +/- 2°C or overnight at 4 °C.
  - b. Wash the plate 6 to 10 times with PSTB-T 1X.
6. Add the antibody + conjugate
  - a. Mix the monoclonal antibody (Bottle A) with the enzymatic conjugate (Bottle B) in ECI Buffer in a 1:200<sup>8</sup> proportion for both antibodies. From this mix, 100  $\mu$ l is poured in each well and the plate is kept in a wet chamber for 2 hours 37+/- 2°C.
  - b. Dissolve in 1000 ml of phosphate buffer solution 1X (PBST):

<b>Conjugate solution (ECI)</b>	
Bovine serum albumin (BSA)	2.0 g
Poly vinyl pyrrolidinone (PVP) Molecular weight 24-40,000	10.0 g
Sodium azide	0.2 g
Adjust the pH to 7,4 (+/- 0,2), store at 4°C.	

- c. Wash the plate 6 to 10 times.
7. Addition of substrate.
  - a. Prepare the substrate solution from the enzyme diluting the para-nitrophenyl phosphate in extraction grinding buffer in a proportion of 1mg/ml (usually one tablet/5 ml). Place 100  $\mu$ l in all the working wells, including the target and incubate under dark conditions from 15 to 45 minutes.

<b>Buffer solution for PNP (developing)</b>	
Diethanolamine	97.0 ml
Sodium azide	0.2 g
Adjust the final volume to 1 L. with sterile distilled water. Adjust the pH to 9,8 with hydrochloric acid, store at 4°C in refrigeration in amber bottle or wrap in aluminium to protect from light.	

8. Plate evaluation
  - a. Turn on and program the ELISA plate reader.
  - b. Note if there are colors in the wells. Change in color is indicative of positive results.
  - c. Read in the plate reader and print the readings.

<sup>8</sup> Dilution commonly used. Follow the manufacturer's instructions.

- d. Analyze the results and record them in the record book for laboratory samples, writing the date of the response. Record the results in the database.

### Considerations

For a diagnostic with reliable readings, the following controls must be included in the plate:

- Uncovered wells
- Extraction buffer
- Healthy plant (negative control)
- Sick plant (positive control)

Infected plants with different CTV variants can be included<sup>9</sup> as positive controls. A negative control corresponding to a healthy plant of the same species should also be included. In both cases (positive and negative controls) replicates should be included. Positive readings must be twice the value of the negative control. Negative results of unknown samples must present absorbency values similar to the negative controls. If the samples are different than their replicate or have readings 1.5 times the value of the negative control, the diagnostic must be repeated. If the positive controls do not react properly, the diagnostic must also be repeated.

## 3.2. Immunoimpression- ELISA

The preparation of the plant extracts to be diagnosed is the most important limiting factor to detect CTV with conventional ELISA. Therefore, some variants have been developed such as immunoimpression-ELISA, which is a technique that uses capture membranes that do not require sample crushing or homogenization, thus allowing the analysis of thousands of samples in a simple and fast way (Cambra *et al.*, 2000).

The process consists of four basic and sequential stages:

1. Collection and impression of membrane samples.
2. Blocking the pressed membrane and reaction.
3. Washing.
4. Developing and reading the results.

### 3.2.1 Procedure

1. Make transversal or oblique cuts to young shoots<sup>10</sup>, leaves, pedicels, or peduncles of newly ripe fruit with very sharp instruments.
2. Firmly press the cross sectional tissue against the nitrocellulose membrane of 0.45 mm pore, which is used as an immunoabsorbent. Make 2 impressions per shoot, cutting the ends from its base and apex. This will allow making 10 impressions per mature tree. Positive and negative controls should be included.
3. Let the membranes dry for a few minutes and keep them in a dry place and protected from light until they are analyzed.
4. Block the pores from the membrane (and the rest of the surface) with a bovine serum albumin (BSA) solution with a concentration of 1 % in distilled water. The membranes must stay in the blocking solution for 1 hour at room temperature, or for 16 hours if the operation is done at 4 °C. Slightly shake the membrane to soak it and cover it completely with the blocking solution.
5. Add a solution of specific monoclonal antibodies for CTV, marked with the alkaline phosphatase enzyme (0.1 mg/ml) to the membrane soaked with the albumine solution.

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<sup>9</sup> Two mild and two severe.

<sup>10</sup> It is preferable to collect shoots of 10-15 cm from the youngest material available, from various areas around the tree, preferably from the medium-high section of the canopy, which is the area most likely to be visited by aphids.

6. Incubate for 2 or 3 hours at room temperature with the solution completely covering the membrane. Once the reaction time has elapsed, discard the solution of conjugated antibodies.
7. Rinse the membrane<sup>11</sup> with washing buffer (PBS + 0.05% Tween 20)
8. Add the substrate (BCIP-NBT Sigma Fast Tablets)<sup>12</sup> specific for the enzyme (10 tablets in 100 ml of distilled water). Slightly cover the membranes and incubate at room temperature until violet-purple precipitation start to show.
9. When positive controls have shown some color, stop the reaction by washing the membranes with running water. Precipitations will show after 3-7 minutes of incubation at room temperature.
10. Take the reading from the membranes once they are dry. In many cases, a reading at first sight is enough, but they should be observed with a magnifying glass or dissecting microscope.

### 3.3 Biological techniques

Biological indexing is a technique that, under proper conditions, guarantees an accurate diagnosis. Biological indexing (bioindexing) for citrus pathogens is based on the use of citrus indicator plants free of pathogens, which react to the virus infection by expressing diagnostic symptoms depending on the pathogen and isolate (Roistacher, 1991). Each sample (budwood to be tested) is inoculated into replicated indicator plants. Uninoculated indicators are used as healthy (negative) controls and indicators inoculated with tissue known to be infected with mild to severe isolates of the pathogen are used as positive controls. Biological indexing requires appropriate environmental conditions in a greenhouse environment and 1 month to a year to evaluate the material tested, depending upon the pathogen. Bioindexing has been validated as reliably identifying citrus pathogens in mixed infections, which are common in the case of citrus (Vidalakis *et al.*, 2004).

#### 3.3.1. Procedure

The procedures used for biological indexing for CTV are well established (Roistacher, 1991). For the biological indexing of CTV the preferred indicator plant is Mexican lime (*Citrus aurantiifolia*), the small-fruited acid lime. The Mexican lime seedlings must be healthy with trunks of 4-10 mm diameter and 100 cm height. The seedlings of Mexican lime should be planted 3 per pot. Each pot will have 2 inoculated seedlings and 1 non-inoculated. The uninoculated plant will serve as a healthy control. There should be between 4 and 8 replicates from each tree diagnosed and at least 2 positive controls (one strong, one weak) for each test.

From the plants to be tested, take budsticks from a minimum of 4 quadrants of each tree. In the case of field trees where there is a risk of infection, take budsticks from 8 quadrants. This is very important in the case of foundation trees maintained in the field. "Buds" (buds with eyes, blind buds, or chip buds) are grafted two per indicator plant. It is important that the inoculum include phloem tissue and that the phloem of the inoculum and indicator are in good contact since CTV is phloem-limited. If leaf disks are used, a minimum of five or six be used per plant. The blades used to do the graft should be disinfected with a solution of 10 % of commercial sodium hypochloride between samples. Immediately after the inoculation the plants should be cut back to a height of 20-25 cm.

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<sup>11</sup> The washing stage is essential since it eliminates all the antibodies marked with alkaline phosphatase which have not reacted. That way there would only be traces of enzyme on the selections of printed plant material infested with CTV.

<sup>12</sup> Nitro blue tetrazolium and bromo chlorine indolyl phosphate (NBT+BCIP)

Once the plants are inoculated, symptom expression is favored by maintaining temperatures of 24 – 28 °C (day) and 17 – 21 °C (night) for 2 – 4 months. After 2 weeks, the grafting tape should be removed and survival recorded. The side branches of the seedlings should not be pruned for the first three flushes (more or less 8 weeks) in order to obtain the maximum quantity of leaves to observe for symptoms. The majority of inoculated plants will show symptoms within 5 weeks and almost all within 8 weeks. The primary symptom of tristeza is vein clearing in young and mature leaves. This symptom is observed best in leaves held above so that sunlight shines through the leaf. The best time to observe these symptoms is when the leaf just stops increasing in size. In the case of severe strains of tristeza (seedling yellows), the vein clearing can develop into vein corking. Another characteristic symptom of tristeza in Mexican lime is leaf cupping. This is seen when conditions are very good for indexing. It can persist in mature leaves. On the other hand, leaf cupping is also a symptom of vein enation in Mexican lime and so leaf cupping by itself is not diagnostic for tristeza. After 8 weeks, the bark can be removed and stem pitting observed with certain strains of tristeza. However, stem pitting in Mexican lime is better evaluated by removing the side shoots after the third flush (about eight weeks) and training the plants to a single leader. Stem pitting can then be evaluated after four to six months after the initial inoculations.

Indicator plants can also be used to biocharacterize isolates of tristeza (Garnsey et al, 1987; Roistacher, 1991). In addition to the intensity of the reaction in Mexican lime, the isolates can be evaluated for stem pitting (Figure 1g), seedling yellows (Figure 1h), or quick decline. All these symptoms indicate a severe strain that can produce economic consequences. Although various isolates of tristeza can cause stem pitting or seedling yellows in various indicator plants, 'Duncan' grapefruit is preferred for this use. Sour orange can serve as an additional indicator for stem pitting and 'Madame Vinous' or 'Pineapple' sweet orange can show whether a strain causes stem pitting in sweet orange varieties. Thus, five indicators are needed to biocharacterize different isolates of tristeza: Mexican lime (basic symptoms, stem pitting); sour orange (seedling yellows, stunting); sweet orange (stem pitting, stunting); 'Duncan' grapefruit (seedling yellows, stem pitting, stunting); and sweet orange budded on sour orange (phloem necrosis, stunting). The grapefruit and sour orange are grown 3 per pot and the sweet orange 1 per pot for these uses. Observation of decline on sour orange rootstocks has taken 2 – 3 years in the past. Recently, a more rapid method has been developed (Pina *et al.*, 2005) where a lack of growth of sour orange buds grafted onto 'Madame Vinous' sweet orange that has been inoculated with a decline-inducing strain has been correlated with decline-inducing isolates.

#### **4. Record**

A record of tested samples must be kept containing the following:

- Code for the reference number of the sample.
- Variety and origin of the sample.
- Symptoms description (including pictures, as appropriate) or no symptoms.
- Methods used in the diagnostic and the results obtained with each method, including controls (pictures of the diagnostic gel or records of results for the ELISA testing, on which the diagnostic was based)
- Name of the laboratory, and when appropriate, the names of the persons responsible for the diagnostic.

Records and evidences of the diagnostic results should be kept for at least one year.

#### **5. Contacts for additional information**

Centro Nacional de Referencia Fitosanitaria. (CNRF). Dirección General de Sanidad Vegetal. Guillermo Pérez Valenzuela No. 127 Col. Del Carmen, Coyoacán, Del. Coyoacán, México, DF 04100

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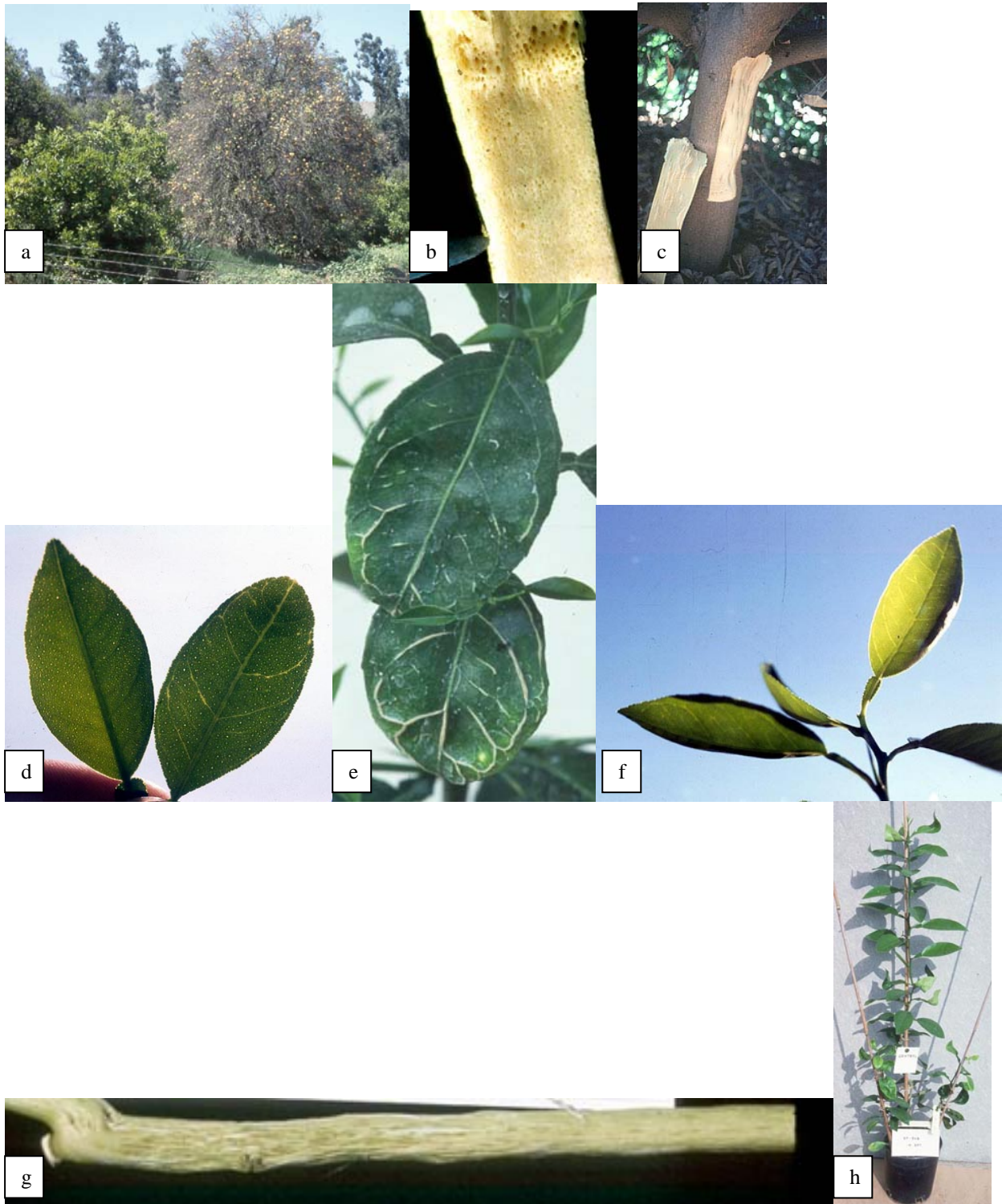


Figure 1. Symptoms of Citrus Tristeza Closterovirus, a) Tristeza decline of sweet orange grown on sour orange rootstock, b) and c) phloem necrosis of bud union of sweet orange – sour orange infected with decline strain of CTV, d) vein clearing in leaves of Mexican lime indicator, e) vein corking in severe (seedling yellows) strain of CTV in Mexican lime indicator, f) Leaf cupping in leaves of Mexican lime indicator, g) stem pitting in Eureka lemon indicator, h) seedling yellows reaction in grapefruit indicator. (All photos by CN Roistacher)

## 7. Approval


### Review


NAPPO Treatment and Diagnostic Protocols are subject to periodic review and amendment. The next review date for this NAPPO protocol is 2014. A review of any NAPPO Protocol may be initiated at any time upon the request of a NAPPO member country.

### Approved

This Protocol was approved by the North American Plant Protection Organization Executive Committee on October 19, 2009 and is effective from this date.

Approved by:

  
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Executive Committee Member  
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