Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

Normes OEPP
EPPO Standards

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

PM 7/31

Organization Européenne et Méditerranéenne pour la Protection des Plantes
1, rue Le Nôtre, 75016 Paris, France

Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Diagnostic Protocols for Regulated Pests are intended to be used by National Plant Protection Organizations, in their capacity as bodies responsible for the application of phytosanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use, etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all diagnostic protocols:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards 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 or that proper positive and negative controls are included.

References

EPPO/CABI (1996) *Quarantine Pests for Europe*, 2nd edn. CAB International, Wallingford (GB).

EU (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

FAO (1997) *International Plant Protection Convention* (new revised text). FAO, Rome (IT).

IPPC (1993) *Principles of plant quarantine as related to international trade*. ISPM no. 1. IPPC Secretariat, FAO, Rome (IT).

IPPC (2002) *Glossary of phytosanitary terms*. ISPM no. 5. IPPC Secretariat, FAO, Rome (IT).

OEPP/EPPO (2003) EPPO Standards PM 1/2 (12): EPPO A1 and A2 lists of quarantine pests. *EPPO Standards PM1 General phytosanitary measures*, 5–17. OEPP/EPPO, Paris.

Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.
Quarantine pest : a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Diagnostic Protocols for Regulated Pests provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Nineteen EPPO standards on diagnostic protocols have already been approved and published. Each standard is numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69
PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77
PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253
PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259
PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265
PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275
PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279
PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292
PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298
PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310
PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256
PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264
PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270
PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280
PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288
PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

Several of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'intercomparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

Diagnostic protocols for regulated pests[1]
Protocoles de diagnostic pour les organismes réglementés

Citrus tristeza closterovirus

Specific scope
This standard describes a diagnostic protocol for *Citrus tristeza closterovirus*.

Specific approval and amendment
This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2003-09.

Introduction

The *Closterovirus Citrus tristeza virus* (CTV) causes one of the most damaging diseases of citrus (Bar-Joseph & Lee, 1989) and is the most economically important pathogen of this crop (Lee & Bar-Joseph, 2000). CTV probably originated in Asia and has been disseminated to almost all citrus-growing countries by movement of infected plant material. Subsequent spread by aphid vectors has created major epidemics. Epidemics of tree losses on sour orange rootstock were first reported from South Africa in the early part of the 20th century, and in Argentina and Brazil in the 1930s following the importation of CTV-infected plants and the efficient aphid vector *Toxoptera citricida*. More than 80 million trees grafted on sour orange (*Citrus aurantium*) rootstock have been killed or rendered unproductive by CTV-induced decline. The losses caused in Argentina (more than 10 million trees), Brazil (more than 6 million trees) and USA (more than 3 million trees) have been reported by Bar-Joseph *et al*. (1989). In Spain alone, more than 40 million trees, mainly sweet orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*) grafted on sour orange, have declined progressively (Cambra *et al*., 2000a). In addition, CTV may cause stem pitting in some citrus cultivars regardless of the rootstock used, leading to significant losses in fruit quality and yield.

As a member of the genus *Closterovirus* (Karasev *et al*., 1995), CTV has virions which are flexuous (2000 × 11 nm in size) and contain a non-segmented, positive-sense, single-stranded RNA genome. The sequence of the CTV genome contains 12 open reading frames (ORFs), potentially encoding at least 17 proteins. ORFs 7 and 8 encode proteins with estimated molecular weights of 27.4 (P27) and 24.9 kDa that have been identified as the capsid proteins. The complete sequence of several CTV isolates has been reported (Pappu *et al*., 1994; Karasev *et al*., 1995; Mawassi *et al*., 1996; Vives *et al*., 1999; Yang *et al*., 1999; Albiach-Marti *et al*., 2000; Suastika *et al*., 2001), including the sequence of a typical mild Spanish CTV isolate (Vives *et al*., 1999).

Transmission

CTV is readily transmitted by graft and, in a semipersistent manner, by the main aphid species visiting citrus: *T. citricida*, *Aphis gossypii*, *Aphis spiraecola* and *Toxoptera aurantii*. *T. citricida* (not yet present in continental Europe or in the Mediterranean Basin) is a much more efficient vector than *A. gossypii*, but epidemic spread has occurred in Spain when *A. gossypii* was the predominant aphid species (Cambra *et al*., 2000a). *A. spiraecola* is not an efficient vector but, since its populations can become so high, it may be a significant factor in CTV spread in some areas. *T. aurantii* apparently transmits only certain CTV isolates (Lee & Bar-Joseph, 2000). Eight aphid species (*T. citricida* not included) have been assayed as vectors of different Mediterranean CTV isolates (Hermoso de Mendoza *et al*., 1984, 1988). *A. gossypii* was always the most efficient vector and transmission efficiencies were up to 78%, whereas *A. spiraecola* and *T. aurantii* had very low efficiencies (0–6%). The spatial and temporal spread of tristeza disease has been studied in European citrus orchards (Cambra *et al*., 1988, 1990a; Gottwald *et al*., 1996, 1997; Cambra *et al*., 2000a). A long time may elapse between the introduction of a primary source of inoculum and the development of a disease epidemic (Garnsey & Lee, 1988).

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Isolate differentiation

Field CTV isolates may vary in pathogenicity and may contain multiple genomic virus variants that can be separated by aphids or graft transmission to different citrus host species. The subisolates segregated in this way can be differentiated by pathogenicity tests in different hosts, by dsRNA patterns (Moreno *et al*., 1993) or serologically using specific monoclonal antibodies (Cambra *et al*., 1993). The monoclonal antibody MCA13 (Permar *et al*., 1990) was described in Florida (US) as specific for severe and CTV decline-inducing isolates. Reaction with MCA13 is not necessarily correlated with the decline of trees in the Mediterranean Basin, but constitutes a good indication of potential aggressiveness of an isolate. There are no molecular methods allowing reliable typing of CTV isolates according to their aggressiveness. It has been demonstrated that the haplotype distribution of two CTV genes can be altered after host change or aphid transmission (Ayllón *et al*., 1999). Molecular hybridization (Albiach *et al*., 1995) and single-strand conformation polymorphisms analysis of the coat protein gene (Rubio *et al*., 1996) have been used to differentiate Mediterranean CTV isolates.

Principal host plants

Most species of *Citrus* and some species in other genera of the family *Rutaceae* (*Aegle marmelos*, *Aeglopsis chevalieri*, *Afraegle paniculata*, *Citropsis gilletiana*, *Microcitrus australis* and *Pamburus missionis*) have been reported as hosts for CTV. Most trifoliate orange clones and many of their hybrids are resistant to infection. Protoplasts of *Nicotiana benthamiana* have been experimentally infected by CTV.

Identity

Name: *Citrus tristeza virus*
Acronym: CTV
Taxonomic position: Viruses, Closteroviridae, *Closterovirus*
Bayer computer code: CTV000
Phytosanitary categorization: EPPO A2 list n°93, EU Annex II/AII for European isolates EU Annex II/AI for non European isolates

Detection

Symptoms

Symptom expression in citrus hosts is highly variable and affected by environmental conditions, host species and the aggressiveness of the CTV isolate. Some CTV isolates are mild and produce no noticeable effect on most commercial citrus species. In general, mandarins are especially tolerant to CTV infection. Sweet orange, sour orange, rough lemon (*C. jambhiri*) and Rangpur lime (*C. limonia*) are usually symptomless but may react to some aggressive isolates. Reactive hosts include lime, grapefruit (*C. paradisi*), some cultivars of pummelo (*C. grandis*), alemow (*C. macrophylla*), some cultivars of sweet orange, some citrus hybrids and some citrus relatives above mentioned. Stunting, leaf cupping, vein clearing and chlorosis, stem pitting, and reduced fruit size are common symptoms of susceptible hosts.

One of the most economically significant symptoms of tristeza disease is the decline of trees grafted on sour orange. Sweet orange, mandarin and grapefruit on sour orange rootstock become stunted, chlorotic and often die after a period of several months or years (slow decline), or some days after the first symptoms (quick decline). The decline results from the effects of the virus on the phloem of the sour orange rootstock just below the bud union. Trees that decline slowly generally have a bulge above the bud union, and inverse pinhole pitting (honey combing) on the inner face of the sour orange bark. Some isolates of the virus do not induce decline symptoms, even in trees on sour orange, for many years.

Aggressive CTV isolates 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 trucks and limbs of adult trees. Deep pits in the wood are present under depressed areas of the bark. Fruit quality and yield are greatly reduced in trees with severe stem pitting. Nevertheless, most CTV isolates are able to cause stem pitting in *C. macrophylla* rootstocks and reduce tree vigour. Symptoms caused by CTV are presented in Web Fig. 1.

Identification

The classic identification procedure for CTV is to graft-inoculate indicator seedlings of Mexican lime (Wallace & Drake, 1951) and observe them for vein clearing, leaf cupping, and stem pitting. Electron and light microscopy can be used to identify CTV particles and inclusions, but DAS-ELISA (Bar-Joseph *et al*., 1979; Cambra *et al*., 1979) has revolutionized diagnosis, making it feasible to test many samples during surveys of large citrus areas, for CTV control in nurseries and for epidemiological studies.

Polyclonal antibodies from antisera were used from 1978 to 1983 for routine ELISA tests. The production of monoclonal antibodies specific to CTV (Vela *et al*., 1986; Permar *et al*., 1990) and others reported by Nikolaeva *et al*. (1996) solved the problems of specificity and increased sensitivity of ELISA tests. A mixture of two monoclonal antibodies (3DF1 and 3CA5) or their recombinant versions (Terrada *et al*., 2000) recognizes all CTV isolates tested from different international collections (Cambra *et al*., 1990b). A detailed description and characterization of these monoclonal antibodies has been summarized (Cambra *et al*., 2000a).

The development of Tissue print-ELISA (Garnsey *et al*., 1993; Cambra *et al*., 2000b) for CTV detection in imprinted sections of plant material on nitrocellulose membranes, allowed the sensitive testing of thousands of samples simply and without the need to prepare extracts.

PCR-based assays have been developed based on immunocapture (Nolasco *et al*., 1993) or print or squash capture (Olmos *et al*., 1996; Cambra *et al*., 2000c). A simple procedure has been described to perform nested PCR in a single closed tube (Olmos *et al*., 1999) which allowed CTV detection in single aphids and in plant tissues. A co-operational PCR system (Co-PCR) using a universal probe for hybridization with PCR products (Olmos *et al*., 2002) has been described, giving sensitivity similar to that of nested PCR.

Sampling

Appropriate sampling is critical for serological or molecular detection of CTV. The standard sample for adult trees involves 5 young shoots (from the last flush) or fruit peduncles, or 10 fully expanded leaves, or 5 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 year from sweet orange, mandarin, lemon and grapefruit in the Mediterranean area, but springtime give the highest CTV titres. A reduced CTV titre is observed in Satsuma mandarin during summer. Consequently, the recommended period for sampling includes all vegetative seasons except summer (July–August in the Mediterranean Basin). Flowers or fruits (when available) are also suitable materials for testing (Cambra *et al*., 2002). Standard sampling for nursery plants involves 2 young shoots or 4 leaves. Samples (shoots, leaf petioles, fruit peduncles and flowers) can be stored at 4 °C for not more than 7 days before processing. Fruits can be stored for 1 month at 4 °C.

Sample preparation

Preparation of tissue prints for testing

Tender shoots, leaf petioles, fruit peduncles or flowers are cleanly cut. The fresh cut sections are carefully pressed against a nitrocellulose membrane (0.45 mm), and the trace or print is allowed to dry for a few minutes. For routine testing, at least two prints should be made per selected shoot or peduncle and one per leaf petiole or flower (see sampling). Printed membranes can be kept for several years in a dry place.

Preparation of plant extracts for testing

About 1 g of plant material is weighed, cut in small pieces and placed in a suitable tube or plastic bag for processing. About 20 volumes of extraction buffer are added and the sample is homogenized in tubes using a Polytron (Kinematica) or similar blender. Alternatively, the sample may be homogenized in the plastic bag, using Homex 6 machine (Bioreba) or any manual roller, hammer, or similar tool. The extraction buffer is phosphate-buffered saline (PBS) pH 7.2–7.4 (Appendix 1) supplemented with 0.2% sodium diethyl dithiocarbamate (DIECA) or 0.2% mercapto-ethanol. Samples for serological testing can be prepared in tubes or in plastic bags. Samples for molecular testing should only be prepared in appropriate individual plastic bags.

Screening tests

Biological testing

The object of testing 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 lime). The indicator is graft-inoculated according to conventional methods and held under standard conditions (Roistacher, 1991), with 4–6 replicates. Symptom onset is compared with that of positive and negative control plants.

Serological tests

Tissue print or immunoprinting ELISA, or direct tissue blot immunoassay (DTBIA), is performed according to Garnsey *et al*. (1993) and Cambra *et al*. (2000b) using the detailed protocol described in Appendix 2 and materials described in Appendix 1.

Double Antibody Sandwich ELISA (DAS-ELISA), in the conventional or biotin/streptavidin system, is performed according to Garnsey & Cambra (1991), using the detailed protocol described in Appendix 2 and materials described in Appendix 1.

Molecular tests

Immunocapture RT-PCR (IC-RT-PCR) is performed according to Wetzel *et al*. (1992), Nolasco *et al*. (1993) and Rosner *et al*. (1998) using the detailed protocol described in Appendix 2 and materials described in Appendix 1.

Immunocapture nested RT-PCR in a single closed tube is performed according to Olmos *et al*. (1999) using the detailed protocol described in Appendix 2 and materials described in Appendix 1.

Reference material

Standard CTV-infected and healthy citrus controls, CTV-specific monoclonal antibodies (in addition to the commercially available ones of Appendix 1) and CTV-specific oligonucleotide primer sequences are available for non-profit institutions from Instituto Valenciano de Investigaciones Agrarias (IVIA), Department Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada (Valencia). Spain.

Possible confusion with similar species

None.

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol and in the decision scheme (Fig. 2) should have been followed and appropriate controls should have been included. When CTV is diagnosed for the first time, or in critical cases (import/export), a combination of two different screening methods should be used, based on biological testing (inoculation of Mexican lime) and on serological or molecular detection (with the validated protocols and reagents).

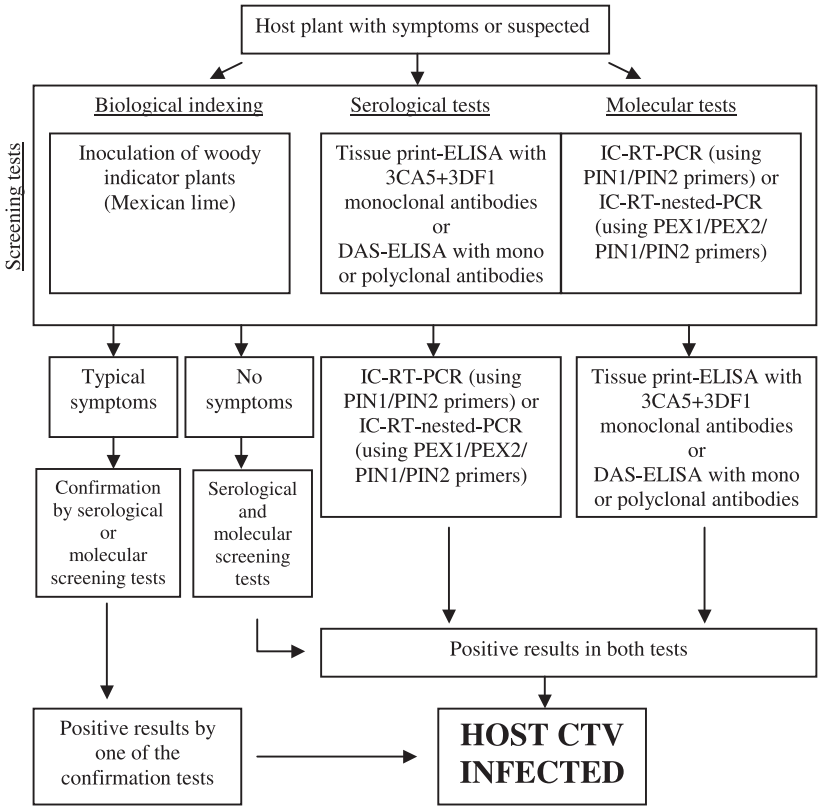

Fig. 2 Detection scheme for the detection and identification of *Citrus tristeza closterovirus*.

Report on the diagnosis

The report on the execution of the protocol should include:

- results obtained by the recommended procedures
- information and documentation on the origin of the infected material
- a description of the disease symptoms (with photographs if possible)
- an indication of the magnitude of the infection
- comments as appropriate on the certainty or uncertainty of the identification.

The original sample (with labels, if applicable) should be kept under proper conditions as long as possible. Sample extract and PCR amplification product should be kept at −80 °C for 3 months (or longer for legal purposes). Printed tissue sections on nitrocellulose (see sample preparation) and the developed membrane after reading should be kept at room temperature for 6 months.

Further information

Further information on this organism can be obtained from: Instituto Valenciano de Investigaciones Agrarias (IVIA), Department Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada (Valencia). Spain. E-mail: *mcambra@ivia.es*.

Acknowledgements

This protocol was originally drafted by: M. Cambra, A. Olmos and M. T. Gorris, Instituto Valenciano de Investigaciones Agrarias (IVIA), Department Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada (Valencia). Spain.

Tel. #34 961391000 Fax #34 961390240

This protocol was ring-tested in different European laboratories[2].

References

Albiach MR, Rubio L, Guerri J, Moreno P, Laigret F & Bové JM (1995) [Differentiation of races of Citrus tristeza virus by molecular hybridization.]

[2]C. Varveri (Benaki Phytopathological Institute, GR); D. Boscia & O. Potere (Istituto di Virologia Vegetale del CNR, Bari, IT); K. Djelouah & A. M. D'Onghia (Istituto Agronomico Mediterraneo, Valenzano, IT); R. Flores & C. Muñoz Noguera (Laboratorio de Sanidad Vegetal de Sevilla, ES); M. A. Cambra & M. L. Palazón (Centro de Protección Vegetal, Zaragoza, ES); J. Serra-Aracil (Servicio de Sanidad y Certificación, Valencia, ES); P. Moreno, R. Albiach-Martí & M. E. Martínez (Instituto Valenciano de Investigaciones Agrarias, Laboratorio de Virologia, ES); E. Bertolini & M. C. Martínez (Instituto Valenciano de Investigaciones Agrarias Laboratorio de Serología, ES); M. T. Gorris & A. Olmos (Instituto Valenciano de Investigaciones Agrarias Laboratorio de Virología e Immunología, ES).

Investigacion Agraria. Producción Y Protección Vegetales **10**, 263–274 (in Spanish).

Albiach-Marti MR, Mawassi M, Gowda S, Satyanarayana T, Hilf ME, Shanker S, Almira EC, Vives MC, Lopez C, Guerri J, Flores R, Moreno P, Garnsey SM & Dawson WO (2000) Sequences of Citrus tristeza virus separated in time and space are essentially identical. *Journal of Virology* **74**, 6856–6865.

Ayllón MA, Rubio L, Moya A, Guerri J & Moreno P (1999) The haplotype distribution of two genes of citrus tristeza virus is altered after host change or aphid transmission. *Virology* **255**, 32–39.

Bar-Joseph Garnsey SM, Gonsalves D, Moscovitz M, Purcifull DE, Clark MF & Loebenstein G (1979) The use of enzyme-linked immunosorbent assay for detection of citrus tristeza virus. *Phytopathology* **69**, 190–194.

Bar-Joseph M & Lee RF (1989) Citrus tristeza virus. *AAB Descriptions of Plant Viruses* no. 353. AAB, Wellesbourne (GB).

Bar-Joseph M, Marcus R & Lee RF (1989) The continuous challenge of citrus tristeza virus control. *Annual Review of Phytopathology* **27**, 291–316.

Cambra M, Camarasa E, Gorris MT, Garnsey SM, Gumpf DJ & Tsai MC (1993) Epitope diversity of isolates of citrus tristeza virus (CTV) in Spain. *Proceedings 12th International Conference of the Organization of Citrus Virologists*, pp. 33–38. IOCV, Riverside (US).

Cambra M, Garnsey SM, Permar TA, Henderson CT, Gumph D & Vela C (1990b) Detection of citrus tristeza virus (CTV) with a mixture of monoclonal antibodies. *Phytopathology* **80**, 103.

Cambra M, Gorris MT, Marroquín C, Román MP, Olmos A, Martínez MC, 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 MT, Olmos A, Martínez MC, Román MP, Bertolini E, López A & Carbonell EA (2002) European Diagnostic Protocols (DIAGPRO) for citrus tristeza virus in adult trees. *Proceedings 15th International Conference of the Organization of Citrus Virologists*, in press. Riverside (US).

Cambra M, Serra J, Bonet JC & Villalba D (1990a) [Situation of tristeza in Comunidad Valenciana.] *Generalidad Valenciana, Conselleria d'Agricultura i Pesca. Serie Fullets Divulgació no*. 3–90. Valencia (ES) (in Spanish).

Cambra M, Gorris MT, Román MP, Terrada E, Garnsey SM, Camarasa E, Olmos A & Colomer M (2000b) Routine detection of citrus tristeza virus by direct Immunoprinting-ELISA method using specific monoclonal and recombinant antibodies. *Proceedings 14th International Conference of the Organization of Citrus Virologists*, pp. 34–41. IOCV, Riverside (US).

Cambra M, Olmos A, Gorris MT, Marroquín C, Esteban O, Garnsey SM, Llauger R, Batista L, Peña I & Hermoso de Mendoza A (2000c) Detection of citrus tristeza virus by print capture and squash capture-PCR in plant tissues and single aphids. *Proceedings 14th International Conference of the Organization of Citrus Virologists*, pp. 42–48. IOCV, Riverside (US).

Cambra M, Moreno P & Navarro L (1979) [Rapid detection of *Citrus tristeza virus* by ELISA.] *Anales INIA, Serie Protección Vegetal* **12**, 115–125 (in Spanish).

Cambra M, Serra J, Villalba D & Moreno P (1988) Present situation of the citrus tristeza virus in the Valencian Community. *Proceedings 10th International Conference of the Organization of Citrus Virologists*, pp. 1–7. *IOCV*, Riverside (US).

Garnsey SM & Cambra M (1991) Enzyme-linked immunosorbent assay (ELISA) for citrus pathogens. In: *Graft-Transmissible Diseases of Citrus. Handbook for Detection and Diagnosis* (Ed. Roistacher CN), pp. 193–216. FAO, Rome (IT).

Garnsey SM & Lee RF (1988) Tristeza. In: *Compendium of Citrus Diseases* (Ed. Whiteside JO, Garnsey SM & Timmer LW), pp. 48–50. APS Press, St Paul (US).

Garnsey SM, Permar TA, Cambra M & Henderson CT (1993) Direct tissue blot immunoassay (DTBIA) for detection of citrus tristeza virus (CTV). *Proceedings 12th International Conference of the Organization of Citrus Virologists*, pp. 39–50. IOCV, Riverside (US).

Gottwald TR, Cambra M, Moreno P, Camarasa E & Piquer J (1996) Spatial and temporal analyses of citrus tristeza virus in eastern Spain. *Phytopathology* **86**, 45–55.

Gottwald TR, Garnsey SM, 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.

Hermoso de Mendoza A, Ballester-Olmos JF & Pina JA (1984) Transmission of citrus tristeza virus by aphids (Homoptera, Aphididae) in Spain. *Proceedings 9th International Conference of the Organization of Citrus Virologists*, pp. 23–27. IOCV, Riverside (US).

Hermoso de Mendoza A, Ballester-Olmos JF & Pina JA (1988) Comparative aphid transmission of a common citrus virus isolate and a seedling yellows-citrus tristeza virus isolate recently introduced in Spain. *Proceedings 12th International Conference of the Organization of Citrus Virologists*, pp. 39–50. IOCV, Riverside (US).

Karasev AV, Boyko VP, Gowda S, Nikolaeva OV, Hilf ME, Koonin EV, Niblett CL, Cline K, Gumpf DJ, Lee RF, Garnsey SM & Dawson WO (1995) Complete sequence of the citrus tristeza virus RNA genome. *Virology* **208**, 511–520.

Lee RF & Bar-Joseph M (2000) Tristeza. In: *Compendium of Citrus Diseases*, 2nd edn (Ed. Timmer LW, Garnsey SM & Graham JH), pp. 61–63. APS Press, St Paul (US).

Mawassi M, Mietkiewska E, Gofman R, Yang G & Bar JM (1996) Unusual sequence relationships between two isolates of citrus tristeza virus. *Journal of General Virology* **77**, 2359–2364.

Moreno P, Guerri J, Ballester-Olmos JF, Albiach R & Martínez ME (1993) Separation and interference of strains from a citrus tristeza virus isolate evidenced by biological activity and double-stranded RNA (dsRNA) analysis. *Plant Pathology* **42**, 35–41.

Nikolaeva OV, Karasev AV, Powell CA, Gumpf DJ, Garnsey SM & Lee RF (1996) Mapping of epitopes for citrus tristeza virus-specific monoclonal antibodies using bacterially expressed coat protein fragments. *Phytopathology* **86**, 974–979.

Nolasco G, de Blas C, Torres V & Ponz F (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the routine diagnosis of plant viruses and subviral pathogens. *Journal of Virological Methods* **45**, 201–218.

Olmos A, Bertolini E & Cambra M (2002) Simultaneous and co-operational amplification (Co-PCR): a new concept for detection of plant viruses. *Journal of Virological Methods* **106**, 51–59.

Olmos A, Cambra M, Esteban O, Gorris MT & 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.

Olmos A, Dasí MA, Candresse T & Cambra M (1996) Print capture PCR: a simple and highly sensitive method for the detection of plum pox virus (PPV) in plant tissues. *Nucleic Acids Research* **24**, 2192–2193.

Pappu HR, Karasev AV, Anderson EJ, Pappu SS, Hilf ME, Febres VJ, Eckloff RM, McCaffery M, Boyko V, Gowda S, Dolja VV, Koonin EV, Gumpf DJ, Cline KC, Garnsey SM, Dawson WO, Lee RF & Niblett CL (1994) Nucleotide sequence and organization of eight-3′ open reading frames of the citrus tristeza closterovirus genome. *Virology* **199**, 35–46.

Permar TA, Garnsey SM, Gumpf DJ & Lee R (1990) A monoclonal antibody that discriminates strains of citrus tristeza virus. *Phytopathology* **80**, 224–228.

Roistacher CN (1991) *Graft-transmissible Diseases of Citrus. Handbook for Detection and Diagnosis*. FAO, Rome (IT).

Rosner A, Shilboleth Y, Spiegel S, Krisbai L & Kölber M (1998) Evaluating the use of immunocapture and sap-dilution PCR for the detection of *Prunua Necrotic Ringspot Virua. Acta Horticulturae* no. 472, 227–233.

Rubio L, Ayllón MA, Guerri J, Pappu H, Niblett CL & Moreno P (1996) Differentiation of citrus tristeza closterovirus (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *Annals of Applied Biology* **129**, 479–489.

Suastika G, Natsuaki T, Terui H, Kano T, Ieki H & Okuda S (2001) Nucleotide sequence of *Citrus tristeza virus* seedling yellows isolate. *Journal of General Plant Pathology* **67**, 73–77.

Terrada E, Kerschbaumer RJ, 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.

Vela C, Cambra M, Cortés E, Moreno P, Miguet 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.

Vives MC, Rubio L, Lopez C, Navas-Castillo J, Albiach-Marti MR, Dawson WO, Guerri J, Flores R & Moreno P (1999) The complete genome sequence of the major component of a mild citrus tristeza virus isolate. *Journal of General Virology* **80**, 811–816.

Wallace JM & Drake RJ (1951) Recent developments in studies of quick decline and related diseases. *Phytopathology* **41**, 785–793.

Wetzel T, Candresse T, Macquaire G, Ravelonandro M & Dunez J (1992) A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. *Journal of Virological Methods* **39**, 27–37.

Yang ZN, Mathews DM, Dodds JA & Mirkov TE (1999) Molecular characterization of an isolate of citrus tristeza virus that causes severe symptoms in sweet orange. *Virus Genes* **19**, 131–142.

Appendix 1. Materials

Standard CTV-infected and healthy citrus controls and CTV specific monoclonal antibodies (in addition to the commercially available ones indicated below) are available for non-profit purposes at Instituto Valenciano de Investigaciones Agrarias (IVIA). Carretera de Moncada-Náquera km 5. 46113 Moncada (Valencia). Spain.

Materials for serological testing

Tissue print-ELISA

A complete kit (validated in ring tests) based on 3DF1 + 3CA5 CTV-specific monoclonal antibodies, including preprinted membranes with positive and negative controls and all reagents, buffers and substrate, is available from PLANT PRINT Diagnòstics, S.L., De la March 36, Bajo, 46512 Faura, Valencia. Spain. E-mail: plantprint@wanadoo.es

DAS-ELISA kits

Complete kits based on 3DF1 + 3CA5 specific monoclonal antibodies to CTV are commercially available, for the DAS-ELISA biotin/streptavidin system, from: 1) INGENASA (validated in ring tests), Hermanos García Noblejas 41, 2ª planta, 28037 Madrid (ES), *http://www.ingenasa.es*; (2) REAL, CE Durviz S.L., Parque Tecnológico de Valencia, Leonardo Da Vinci, 10, 46980 Paterna (Valencia) (ES), *http://www.durviz.com*. For conventional DAS-ELISA, they are available from: Agdia Incorporated, 30380 County Road 6, 46514 Elkart (US). *http://www.agdia.com*

Complete kits based on polyclonal antibodies to CTV are commercially available from: (1) Adgen Limited, Nellies Gate, Auchincruive, Ayr KA6 5HW (GB), *http://www.adgen.co.uk*; (2) BIORAD Laboratories-SANOFI, Rue Raimond Poincaré 3-BD, 92430 Marnes La Coquette (FR), *http://www.bio-rad.com*; (3) Bioreba, Chr. Merian-Ring 7, CH 4153 Reinach BL1 (CH), *http://www.bioreba.ch*. Streptavidin alkaline phosphatase-linked ELISA (Cat no. 1089 161) is available from Roche Diagnostics GmbH, Mannheim (DE).

Buffers

PBS, pH 7.2–7.4: NaCl 8 g; KCl 0.2 g; $Na_2HPO_4 \cdot 12H_2O$ 2.9 g; KH_2PO_4 0.2 g; distilled water 1 L.

Carbonate buffer pH 9.6: Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; distilled water 1 L.

Washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20): NaCl 8 g; KCl 0.2 g; $Na_2HPO_4 \cdot 12H_2O$ 2.9 g; KH_2PO_4 0.2 g; Tween 20 500 µL; distilled water 1 L.

Colorimetric substrate buffer for alkaline phosphatase: diethanolamine 97 mL; dilute in 800 mL of distilled water. Adjust to pH 9.8 with concentrated HCl and make up to 1000 mL with distilled water.

Precipitating substrate buffer for alkaline phosphatase: Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (BCIP-NBT) – Cat No. –B-5655 – Sigma Aldrich GmbH, Stenheim (DE).

Materials for molecular tests

Oligonucleotide primer sequences (validated in ring-test):
PEX1: 5′–3′ TAA ACA ACA CAC ACT CTA AGG
PEX2: 5′–3′ CAT CTG ATT GAA GTG GAC
PIN1: 5′–3′ GGT TCA CGC ATA CGT TAA GCC TCA CTT
PIN2: 5′–3′ TAT CAC TAG ACA ATA ACC GGA TGG GTA

Buffers

Carbonate buffer pH 9.6: Na_2CO_3 1.59 g; $NaHCO_3$ 2.93 g; distilled water 1 L.

Washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20): NaCl 8 g; KCl 0.2 g; $Na_2HPO_4 \cdot 12H_2O$ 2.9 g; KH_2PO_4 0.2 g; Tween 20 500 µL; distilled water 1 L.

50X TAE buffer: Tris 242 g; 0.5 M Na_2EDTA pH 8.0 100 mL; glacial acetic acid 57.1 mL; distilled water to 1 L.

Loading buffer: 0.25% bromophenol blue; 30% glycerol in H_2O.

Appendix 2. Detailed protocols

Tissue print ELISA

The method follows Garnsey *et al*. (1993) and Cambra *et al*. (2000b). Prepare 1% solution of bovine serum albumin (BSA) in distilled water. Place the membranes (recommended size about 7 × 13 cm) in an appropriate container (tray, hermetic container, plastic bag …). Cover with albumin solution and incubate for 1 h at room temperature, or overnight at 4 °C. Slight agitation is recommended during this step. Discard the albumin solution and keep the membranes in the same container.

Prepare a solution of CTV-specific 3DF1 + 3CA5 monoclonal antibodies linked to alkaline phosphatase (about 0.1 µg mL^{-1} of each monoclonal antibody in PBS) (Appendix 1) or of

3DF1 scFv-AP/S + 3CA5 scFv-AP/S fusion proteins expressed in *E. coli* (appropriate dilution in PBS). Pour onto the membranes, covering them and incubate for 2–3 h at room temperature, then discard the conjugate solution. Rinse the membranes and the container with washing buffer (Appendix 1). Wash by shaking (manually or mechanically) for 5 min. Discard the washing buffer and repeat the process twice. Pour the alkaline phosphatase substrate buffer over the membranes (Appendix 1) and incubate until a purple-violet colour appears in positive controls (about 10–15 min). Stop the reaction by washing the membranes with tap water. Spread the membranes on absorbent paper and let them dry.

Observe the prints using a low-power magnification (X10–X20). Presence of purple-violet precipitates in the vascular region of plant material reveals the presence of *Citrus tristeza virus*.

DAS-ELISA

The method follows Garnsey & Cambra (1991), by the conventional or biotin/streptavidin systems. Prepare an appropriate dilution of polyclonal antibodies or monoclonal antibodies 3DF1 + 3CA5 (Appendix 1) (usually 1–2 μg mL^{-1}) in carbonate buffer pH 9.6 (Appendix 1). Add 200 μL to each well. Incubate at 37 °C for 4 h or at 4 °C for 16 h. Wash the wells three times with PBS-Tween (washing buffer) (Appendix 1).

Add 200 μL per well of the plant extract (see sample preparation). Use two wells of the plate for each sample or positive controls and at least two wells for negative controls. Incubate at 4 °C for 16 h. Wash as before. Prepare specific polyclonal or monoclonal antibodies (3DF1 + 3CA5) linked with alkaline phosphatase or biotin (Appendix 1) at appropriate dilution (about 0.1 μg mL^{-1} in PBS with 0.5% bovine serum albumin-BSA added). Add 200 μL to each well. Incubate at 37 °C for 3 h. Wash as before. If antibodies are linked with biotin, use an appropriate dilution of streptavidin-alkaline phosphatase conjugated (Appendix 1). Add 200 μL to each well. Incubate at 37 °C for 30 min and wash as before.

For both methods (conventional or biotin/streptavidin), prepare 1 mg mL^{-1} alkaline phosphatase solution (p-nitrophenyl phosphate) in substrate buffer. Add 200 μL to each well. Incubate at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA test is negative if the absorbance of the sample is less than twice the absorbance of the healthy control, and positive if the absorbance of the sample is equal to or greater than twice that value.

IC-RT-PCR

Immunocapture phase (IC)

Immunocapture follows Wetzel *et al*. (1992), Nolasco *et al*. (1993) or Rosner *et al*. (1998). Prepare a dilution (1 μg mL^{-1}) of CTV-specific polyclonal antibodies or a dilution (0.5 μg mL^{-1} + 0.5 μg mL^{-1}) of monoclonal antibodies (3DF1 + 3CA5) in carbonate buffer pH 9.6 (Appendix 2). Dispense 100 μL of the diluted antibodies into the Eppendorf tubes. Incubate at 37 °C or on ice for 3 h. Wash twice with 150 μL of sterile washing buffer (Appendix 1).

Clarify 100 μL plant extract previously obtained (see extract preparation) by centrifugation (5 min at 13 000 rev min^{-1}), and submit sample to an Immunocapture phase for 2 h on ice (Rosner *et al*., 1998) or alternatively at 37 °C (Wetzel *et al*., 1992), in coated Eppendorf tubes. After the immunocapture phase, wash Eppendorf tubes three times with 150 μL of sterile washing buffer.

Amplification by RT-PCR

CTV detection PIN1-PIN2 primers Olmos *et al*., 1999) (Appendix 2):
PIN1: 5′–3′ GGT TCA CGC ATA CGT TAA GCC TCA CTT
PIN2: 5′–3′ TAT CAC TAG ACA ATA ACC GGA TGG GTA
Cocktail reaction: H_2O 14.30 μL; 10X-Taq Polymerase Buffer 2.5 μL; 25 mM $MgCl_2$ 1.5 μL (1.5 mM); 5 mM dNTPs 1.25 μL (250 μM); 4% Triton X-100 2 μL (0.3%); 25 μM primer PIN1: 1 μL (1 μM); 25 μM primer PIN2 1 μL (1 μM); DMSO 1.25 μL (5%); 10 U μL^{-1} AMV 0.1 μL; 5 U μL^{-1} Taq Polymerase 0.1 μL. Add the 25 μL of cocktail reaction mixture directly to the washed tubes.
Conditions for RT-PCR: 42 °C for 45 min; 92 °C for 2 min; 40 cycles of 92 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; finally 72 °C for 10 min. Hold at 4 °C.

IC nested RT-PCR in a single closed tube

The method follows Olmos *et al*. (1999). Immunocapture phase (IC) as above for IC-RT-PCR.

Amplification by nested RT-PCR

CTV detection PEX1, PEX2, PIN1, PIN2 primers (Olmos *et al*., 1999) (Appendix 1):
PEX1: 5′–3′ TAA ACA ACA CAC ACT CTA AGG
PEX2: 5′–3′ CAT CTG ATT GAA GTG GAC
PIN1: 5′–3′ GGT TCA CGC ATA CGT TAA GCC TCA CTT
PIN2: 5′–3′ TAT CAC TAG ACA ATA ACC GGA TGG GTA
The device for compartmentalization of a 0.5-mL Eppendorf tube for nested RT-PCR in a single closed tube is according Olmos *et al*. (1999) (Web Fig. 3).
Cocktail A (dropped in the bottom of the Eppendorf tube): H_2O 15.8 μL; 10X-Taq Polymerase Buffer 3 μL; 25 mM $MgCl_2$ 3.6 μL (3 mM); 5 mM dNTPs 2 μL (300 μM); 4% Triton X-100 2.2 μL (0.3%); 25 μM primer PEX1 0.6 μL (0.5 μM); 25 μM primer PEX2 0.6 μL (0.5 μM); DMSO 1.5 μL (5%); 10 U μL^{-1} AMV 0.2 μL; 5 U μL^{-1} Taq Polymerase 0.5 μL.
Cocktail B (placed in the cone): H_2O 2.6 μL; 10X-Taq Polymerase Buffer 1 μL; 25 μM primer PIN1 3.2 μL (8 μM); 25 μM primer PIN2 3.2 (8 μM).
Conditions for RT-PCR: 42 °C for 45 min; 92 °C for 2 min; 25 cycles of 92 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min. After this first step, vortex the tube and centrifuge (6000 $g \times 5$ s) to mix cocktail B with products of first amplification. Place the tubes on the thermal cycler and proceed as follows: 40

cycles of 92 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; finally 72 °C for 10 min.

Electrophoresis of PCR products

Prepare 2% agarose gel in TAE buffer 0.5 × (Appendix 2). Place droplets of about 3 µL of loading buffer (Appendix 2) on parafilm, mix 20 µL of PCR product by gentle aspiration with the pipette before loading. Load wells of gel and include positive and negative controls. Include DNA marker 100 bp in the first well of the gel. Run the gel for 20 min at 120 V (medium gel tray: 15 × 10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15 × 25 cm). Soak the gel in ethidium bromide solution for 20 min. Visualize the amplified DNA fragments by UV *trans*-illumination. Observe specific amplicons of 131 bp.

Fig. 1. Symptoms caused by *Citrus tristeza closterovirus*.
A: Chlorotic and declining sweet orange trees grafted on sour orange rootstock infected by CTV, compared with a looking-healthy tree in the middle (picture from Dr. M. Cambra, IVIA, Spain), **B:** Tristeza-induced quick decline of a sweet orange tree on sour orange rootstock in the middle, surrounded by trees in different states of slow decline (picture from Dr. M.Cambra, IVIA, Spain), **C and D**: Bud-union of sweet orange CTV-infected tree grafted on sour orange rootstock, and pin-holing or honeycombing in the inner face of the bark of the sour orange rootstock below the bud union of the tristeza-infected tree (pictures kindly provided by Drs. L. Navarro and P. Moreno, IVIA, Spain), **E, F and G**: Tristeza aggressive isolate-induced small fruits (compared with a normal fruit on the hand) and stem pitting in branches and trunk of a grapefruit tree in Uruguay (pictures from Dr. M. Cambra, IVIA, Spain).

Fig. 3.. Device for compartmentalisation of a 0.5 ml Eppendorf tube for nested RT-PCR in a single closed tube, according Olmos *et al.* (1999).