European Diagnostic Protocols (DIAGPRO) for *Citrus tristeza virus* in Adult Trees

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ABSTRACT. DIAGPRO is a European Union (EU)-funded project to develop and validate European schemes for detection and diagnosis of a broad range of important organisms harmful to plants, according the EU Plant Health Directive (2000/29 as amended). *Citrus tristeza virus* (CTV) is included, and here are reported the results of a study to compare and standardize diagnostic methods for this virus. The most appropriate protocols were found to be graft inoculation of Mexican lime, sweet orange and grapefruit seedlings, DAS and DASI-ELISA, tissue print-ELISA, immunocapture RT-PCR and immunocapture nested RT-PCR in a single closed tube (conventional, print and squash capture formats). Samples of adult trees were tested during the different seasons (springtime gives the highest CTV titers) using five young shoots or fruit peduncles or 10 fully-expanded leaves. Five flowers or fruits per tree, when available, were also found appropriate for reliable detection in recently infected adult trees. Different commercial polyclonal antibodies and the mixture of monoclonal antibodies (MCAs) 3DF1 and 3CA5 are recommended after calibration for conventional ELISA; the same MCAs are recommended for tissue print ELISA, and the primers of Olmos et al., 1999 (NAR 27, 1564-1565) for RT-PCR variants. A combination of two selected methods based on biological indexing, serological or molecular detection, will be required to officially declare a positive detection. Tissue print-ELISA using specific MCAs was found to be the most reliable, simple and economic method for routine analysis of plant material. The protocols and reagents have been ring tested and are published at http://www.csl.gov.uk/prodserve/know/diagpro/ctv.pdf, an Internet website for comments before publication in the EU Official Bulletin.

DIAGPRO is a European Union (EU) funded project to develop and standardize throughout the EU protocols for detection and diagnosis of a broad range of organisms causing major harm to plants (9). In order to detect and identify the organisms, this project coordinated by the Central Science Laboratory (CSL, York, UK), is developing these protocols, using validated user-friendly methods, for approval by the EU Plant Health Standing Committee. *Citrus tristeza virus* (CTV), the causal agent of tristeza disease is included in the project, and consequently appropriate methods have been selected and optimized, and those with greatest potential tested in pilot studies at Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain. Selected protocols, methods and reagents have also been compared and validated in EU-wide 'ring tests', involving parallel testing in a number of laboratories. The resulting protocols will be sent to selected experts for comment before being submitted to the EU Plant Health Standing Committee (13). The protocols are also published on the internet at http://www.csl.gov.uk and form a basis for European and Mediterranean Plant Protection Organization (EPPO) standards (21).

In this paper the different methods for CTV are included, and compared for its routine detection in plant material. The proposed scheme combines different approaches, biological, serological or molecular. Sampling as a critical step has been carefully studied in adult citrus trees recently infected by CTV.

MATERIALS AND METHODS

Sampling season and selection of plant material. Three adult CTV-infected trees of five different citrus species growing in experimental plots at IVIA were selected to evaluate CTV titers in four different seasons in the course of one year. The species, grafted on

Troyer citrange were: Washington Navel sweet orange, Nules Clementine mandarin, Satsuma mandarin, Verna lemon and Marsh grapefruit. Five shoots approx. 10 cm long and two fully-expanded leaves per shoot (total 10 leaves) and three fruit peduncles were systematically collected from around the tree canopy at approximately 2 m height.

The trees were sampled on May 18th, September 7th, November 25th and January 20th of 2000-2001 and analyzed by semi-quantitative DASc-ELISA (2, 3) using the same freeze-dried positive and negative controls in all ELISA plates and the mixture of 3DF1 and 3CA5 CTV-specific monoclonal antibodies (MCAs) (5, 6) from Ingenasa (Madrid). The plates were automatically read when the internal positive control reached an OD of 3.2 at 405 nm.

ELISA values at different seasons, using the different citrus species, and with differing materials (shoots, leaves and peduncles) as grouping factors, and individual trees as within factor, were analyzed statistically using a repeated-measurements analysis of variance (15) due to the correlation between the successive sampling dates of the same trees (dependence assumption). Results using the Greenhouse-Geisser or the Huynh-Feldt adjustments (12, 14) were almost identical to those using the standard analysis of variance (without the dependence assumption), so the latter was used. In addition the ELISA values of flowers collected in springtime was compared with values obtained from albedos of fruits collected in November-December.

Determination of the minimum number of samples per tree for reliable CTV detection. The number of CTV-positive shoots (out of 10) and CTV-positive leaves (out of 20) was evaluated by Tissue print-ELISA (4, 6, 7, 11) in 48 20-yr-old Washington navel sweet orange field trees grafted on Troyer citrange in which infection was recently detected. An infection is termed "recently infected" when a tree tested Tissue print-ELISA negative in November but positive in the following November, assuming that the infection occurred in the interval. The probability of finding CTV in any shoot or leaf was estimated by an iterative procedure based on the binomial distribution of the number of shoots or leaves detected CTV-positive in those adult trees. In addition, the number of CTV-positive shoots (out of five) or leaves (out of 10) was determined in 127 open-field-cultivated adult Nules Clementine mandarin trees detected to be CTV-infected at least 2 yr previously, and in 208 mandarins of the same cultivar, recently CTV infected (as described above).

Biological indexing. Over the years, experience has shown that to detect CTV and evaluate its aggressiveness, seedlings of a minimum of three species (Mexican lime, sweet orange and grapefruit) must be graft-inoculated and held under standard conditions (19).

Serological methods and antibodies. Different ELISA variants were selected from the literature and evaluated. These methods include DAS and DASI-ELISA using plant extracts, and tissue print, immunoprinting or direct tissue blot immunoassay (DTBIA).

Extracts from samples of 52 healthy and CTV-infected Nules Clementine mandarins were prepared by grinding in a Polytron (Kinematica), diluted 1/20 (w/v) in PBS, pH 7.4 plus 0.2% DIECA. The extracts were analyzed in Polysorp (Nunc) microplates using different commercial DAS-ELISA kits based on polyclonal antibodies or the monoclonals 3DF1 and 3CA5 (6, 20) in a mixture for universal detection of any CTV isolate (5). Conventional DAS-ELISA (10) kits with polyclonal alkaline phosphatase-conjugated antibodies were from Adgen, Bioreba and Biorad (Sanofi), and DAS-ELISAs also used alkaline

phophatase-conjugated MCAs from Agdia or biotinylated ones from Ingenasa. The same samples were analyzed by DASI-ELISA (3) and by Tissue print-ELISA (4, 11) using a validated protocol (7) commercially available as a complete kit (Plant Print Diagnostics S.L.) based on MCAs 3DF1 and 3CA5 (5, 6, 20).

Molecular procedures. Amplification of CTV RNA was assayed by RT-PCR in samples from 64 Nules Clementine trees. Different systems of viral target preparation for RT-PCR were based on immunocapture (IC-PCR) (16), or print or squash capture (PC or SC-PCR) (8, 18) which avoids the need to prepare extracts, or previous extraction of nucleic acids with the commercial RNeasy kit (Qiagen). Extracts were prepared by pulping the plant material with a hammer in individual plastic bags lined with a heavy net (Plant Print Diagnostics S.L.) together with extraction buffer (PBS, pH 7.4 + 0.2% DIECA). For RT-PCR, different pairs of primers (8, 16, 17) were compared for detection of different CTV isolates from the IVIA Collection. Nested RT-PCR in a single closed tube was also performed (8, 17) using the print and immunocapture systems.

The sensitivities of IC-RT-PCR and IC-RT-nested PCR were compared using 10-fold serial dilutions of an extract from a CTV-infected Washington navel sweet orange prepared in healthy control extract of the same citrus cultivar.

Evaluation of different selected protocols for CTV detection. The different methods selected were compared in two experiments collecting samples from 20 Nules Clementine mandarins CTV-infected for more than 2 yr as well as samples from 111 recently infected Clementine trees. In the first experiment the samples were blotted or tissue-printed on nitrocellulose membranes for Tissue print-ELISA and on Whatman 3MM paper for PC-RT-nested PCR analysis. Extracts from the same material were prepared and analyzed by IC-RT-nested PCR. In the second experiment five shoots per tree from the 111 Nules Clementine mandarins above described, were collected and analyzed by Tissue print-ELISA and IC-RT-nested PCR in single closed tubes. Trees that gave divergent diagnosis results by the two techniques were tested again 16 days later (Fig. 3). The second analysis was performed by Tissue print-ELISA, IC-RT-nested PCR and by grafting the same shoots (five per tree) on Mexican lime indicator seedlings using two limes per sampled tree. Again, the trees with diagnostic discrepancies were sampled and analyzed for a third time (Fig. 3 summarizes the procedure).

RESULTS

Sampling season, plant material and minimum number of samples per tree for CTV detection. Fig. 1 shows the average of ELISA values obtained with samples collected from different citrus species CTV-infected for more than 2 yr, in four vegetative seasons. Satsuma differed from other species in showing significantly decreased ELISA values in summer. The values in autumn and winter were also lower than for the other citrus species. Nevertheless, all values were clearly positive for all seasons and species. Table 1 shows the analysis of variance for between and within factors. Shoots, leaves or peduncles showed no clear differences in ELISA values

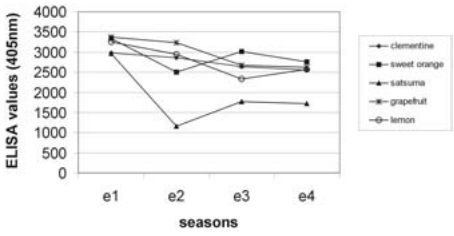

Fig. 1. ELISA values (405 nm) for samples collected from different citrus species in four vegetative seasons.

TABLE 1
REPEATED MEASURES ANALYSIS OF VARIANCE FOR THE ELISA (OD_{405}) VALUES FOR *CITRUS TRISTEZA VIRUS*

Source	DF	Mean Square	Pr > F
Between factors:			
citrus species	4	14001123.05	<0.0001
tree	2	82917.23	0.8694
plant material	6	653144.96	0.3678
error	75	591353.27	
Within factors:			
season	3	8059107.88	<0.0001
season * citrus species	12	1860358.63	<0.0001
season * tree	6	846468.21	0.0175
season * plant material	18	292304.75	0.5693
error (season)	225	321877.65	

for all citrus species except Satsuma where values for leaves and peduncles were higher than shoots. All trees tested gave positive values when flowers or albedo were used. The average of values obtained with all assayed citrus species was 2.973 for flowers in spring and 2.030 for fruit albedo in November-December.

Table 2 shows the number of positive shoots (out of 10) in samples from recently-infected adult sweet orange trees. The number of trees (out of 48) in which only five or fewer shoots tested positive was 39 (81.1%). Only four trees (8.4%) showed all 10 shoots CTV-positive. The number of trees in which only one or two shoots were found positive was 18 (37.4%). Analysis of two leaves collected from each tested shoot, gave identical results.

The probability of finding CTV in any shoot or pair of leaves (out of 10) in a recently infected adult tree was $p = 0.394$. Thus the probability, expressed in percentage, of finding CTV in recently infected trees, sampling progressively more shoots from one to 10 is: 39.4, 63.2, 77.7, 86.5, 91.8, 95.0, 97.0, 98.2, 98.9 and 99.3. Thus if five shoots or 10 leaves were analyzed CTV would be detected in approximately 92% of the recently infected trees. This number of samples per tree was adopted for all subsequent experiments.

A similar analysis of adult Nules Clementine mandarins infected for more than 2 yr revealed that in the majority of the analyzed trees (99.22% of 127) CTV was detected in all shoots and leaves tested. Only in one tree (0.78% of the trees) was one shoot found CTV-negative.

Table 3 shows the percentage of 208 recently infected mandarin trees giving CTV-positive results when testing different numbers of shoots or pairs or leaves. CTV was detected in all shoots (or leaves) analyzed in only 16.34% of the trees.

TABLE 2
NUMBER OF *CITRUS TRISTEZA VIRUS* (CTV)-POSITIVE SHOOTS* (OUT OF 10) IN ADULT SWEET ORANGE TREES RECENTLY INFECTED (LESS THAN 1 YR)

No. + shoots/tree	No. of trees	Percentage
1	8	16.6
2	10	20.8
3	8	16.6
4	6	12.5
5	7	14.6
6	1	2.1
7	2	4.2
8	0	0.0
9	2	4.2
10	4	8.4
	48	100.0

*The same results were obtained using two leaves per shoot.

TABLE 3
NUMBER OF *CITRUS TRISTEZA VIRUS* (CTV)-POSITIVE SHOOTS* (OUT OF 5) IN ADULT NULES CLEMENTINE TREES RECENTLY INFECTED (LESS THAN 1YR)

No. of positive shoots	No. of trees	Percentage
1	59	28.36
2	43	20.68
3	39	18.75
4	33	15.87
5	34	16.34
	208	100.0

*The same results were obtained using two leaves per shoot.

The percentage of tested trees in which only one shoot, out of five, was found CTV-positive was 28.36.

Selected protocols and reagents. The selected protocols and reagents for CTV detection were:

1. Biological indexing using graft inoculation of Mexican lime, sweet orange and grapefruit seedlings according Roistacher's protocol (19).

2. Serological methods: DAS-ELISA using commercial kits based on polyclonal antibodies (Adgen, Bioreba and Biorad) or on the MCAs 3DF1 + 3CA5 (Ingenasa and Agdia), and also the Tissue print or immunoprinting-ELISA commercial kit (Plant Print Diagnostics S.L.) based on 3DF1 + 3CA5 CTV-specific MCAs. All the antibody kits gave similar results (CTV-positive/negative) when 52 Clementine trees were analyzed. Nevertheless, the best results and discrimination between positives and negatives was obtained with the Ingenasa kit followed by the Agdia kit, both based on the same MCAs (data not shown). The higher ELISA values obtained with the Ingenasa kit are quite probably due to the biotin/streptavidin system used. All results obtained by conventional ELISA coincided with those of Tissue print-ELISA using the Plant Print Diagnostics S.L. commercial kit. DASI-ELISA used as control gave similar ELISA values to the Ingenasa and Agdia kits for CTV-positives-negatives (very low background).

3. Molecular methods based on RT-PCR amplification: Primers described for universal CTV detection (8, 17) were validated for IC-RT-PCR and IC or PC or SC-RT-nested PCR in a single closed tube. These primers gave amplification of 23 different CTV isolates from the IVIA collection and showed high sensitivity. IC-RT-nested PCR was at least 100 times more sensitive than the conventional IC-RT-PCR (Fig. 2). The high sensitivity obtained with nested PCR enabled the use of immobilized targets on paper (PC and SC systems). Nevertheless print or squash capture were less sensitive than immunocapture for routine detection. Analysis of 64 Clementine trees gave 53 positives by IC-RT-nested PCR but only 49 by PC-RT-nested PCR. The direct use of purified nucleic acids as targets, instead of IC, PC or SC methods, supplied excellent results in amplification in all experiments performed.

Comparison of different protocols for CTV detection. Different protocols were evaluated for routine detection of CTV in samples from adult Clementine trees. In the experiment with samples from 20 Clementines infected for more than 2 yr, Tissue print-ELISA, IC-RT-nested PCR and PC-RT-nested PCR detected CTV in 100% of the trees, all methods giving the same results. Nevertheless, another experiment with 111 Clementines infected for only 1 yr, showed different results (Fig. 3). Coincidental results were obtained in 89.2% of the trees when analyzed by Tissue print-ELISA and IC-RT-nested PCR. Twelve trees with non-coincidental results were sampled again, inoculated in Mexican lime and analyzed again. The results of the new tests showed the greater reliability of Tissue print-ELISA. Six trees that were found CTV-positive on March 13th by Tissue print-ELISA but negative by IC-

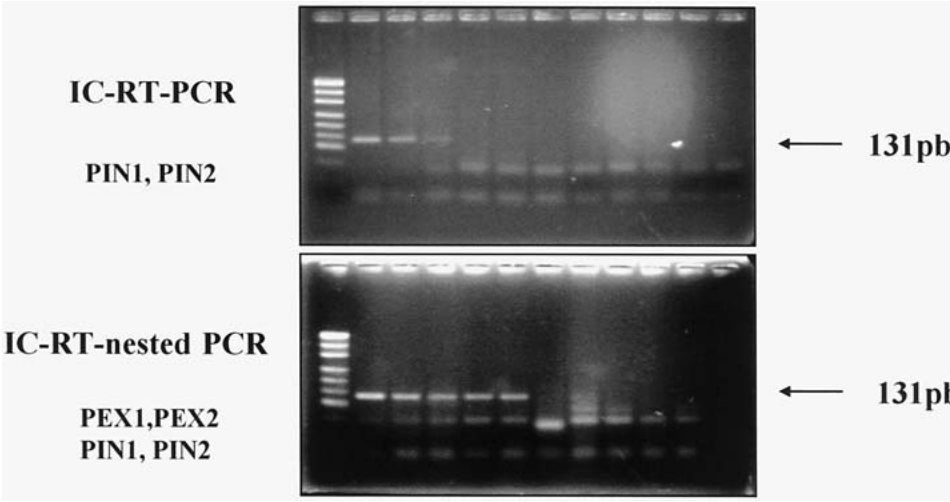

Fig. 2. Comparison of the sensitivity of IC-RT-PCR and IC-RT-nested PCR in a single closed tube using ten-fold serial dilutions of an extract of Washington Navel sweet orange prepared in an extract from healthy sweet orange. The molecular weight of the amplicons (131 bp) was the same in both PCR variants because RT-PCR was performed using primers (PIN1, PIN2) internal to PEX1 and PEX2 used in the first round of amplification.

RT-nested PCR, confirmed again on March 29th the Tissue print-ELISA results. So Tissue print-ELISA results were as before but the IC-RT-nested PCR results were different on the two dates: three trees that were negative by PCR in the first analysis but positive by Tissue print-ELISA were, the second time, positive also by IC-RT-nested PCR. Grafting of the 12 trees to Mexican lime (Fig. 3) confirmed all the Tissue print-ELISA results except one that was negative by grafting. The trees found negative by Tissue print-ELISA remained negative after indexing in Mexican lime in spite of the earlier positive result by IC-RT-nested PCR. A final analysis of the non-coincidental trees analyzed on March 29th (four trees in total), showed total coincidence of the results that finally were as Tissue print-ELISA indicated in the March 29th analysis (three positive, one negative).

DISCUSSION

Analysis of variance showed no clear differences in ELISA values for the different plant tissues and citrus species, except for Satsuma. Flowers or fruit albedo (when available), were also suitable for analysis.

In testing the protocols, sampling was a very important step. Samples (shoots or fully-expanded leaves and peduncles) could be used at any time of year from all citrus cultivars tested, but springtime gave the highest CTV titers, and a reduction in titer was observed in summer with Satsuma. Consequently the recommended period for sampling would include all vegetative seasons avoiding summer (July-August in the Mediterranean Basin).

The probability of finding CTV in any shoot or leaf in the canopy of a recently infected adult tree was approximately of 40%. When five shoots or 10 leaves were analyzed the probability was 92%, and 99.3% if ten shoots or 20 leaves were analyzed. The probability never reaches 100% because it is impossible, routinely, to test all the shoots or leaves in a single tree. A conservative criterion is to sample no less than five shoots or 10 leaves around the can-

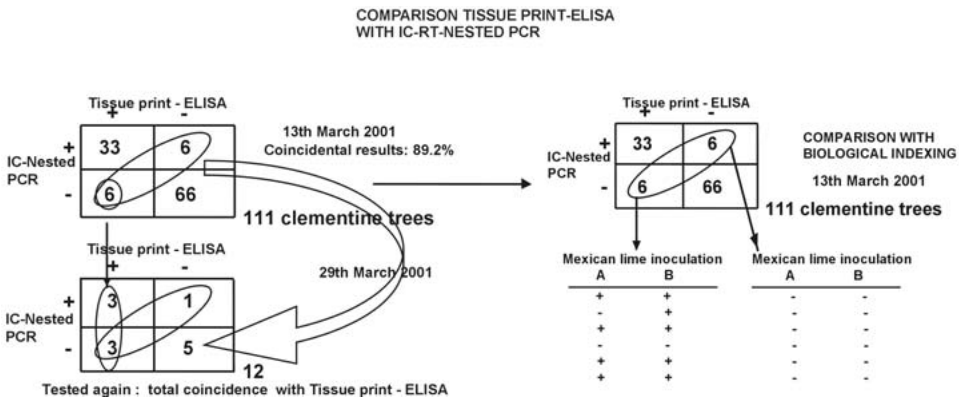

Fig. 3. Procedure and results for the comparison of Tissue print-ELISA versus IC-RT-nested PCR in a single closed tube for detection of CTV in 111 recently infected Clementine trees. Biological indexing on Mexican lime (two repetitions using two plants A and B) was performed to confirm samples with discrepant diagnostic by Tissue print-ELISA and IC-RT-nested PCR, after a first analysis on March 13th.

opy of an adult tree; below this level there is serious risk of not finding CTV in recently infected adult trees.

Sampling five shoots, peduncles or flowers or 10 mature leaves from adult trees infected for more than 2 yr, gave a 99.22% probability of finding CTV, confident enough for routine CTV testing.

CTV-specific primers from the conserved 3' UTR of the CTV genome (8, 17) were shown to be useful to amplify CTV targets by conventional IC-RT-PCR using tissue extracts. Nevertheless RT-nested PCR in a single closed tube is recommended because of its greater sensitivity. The only limitation of this technique is that 0.5 ml Eppendorf tubes are required for the physical separation of the two PCR cocktails based on the described compartmentalization (17). Due to the high sensitivity of RT-nested PCR in a single closed tube, the print or squash methods for sample preparation can be used.

Comparison of Tissue print-ELISA or DTBIA with IC-RT-nested PCR for CTV detection in recently infected Clementine trees, showed the greater reliability of Tissue print-ELISA. Samples from the 12 trees that had given conflicting results on March 13th (Fig. 3) were grafted on Mexican lime seedlings. The final results, obtained after biological indexing and after repeat testing, confirmed the previous Tissue print-ELISA results. An analysis of discrepancies performed some years ago using Washington Navel sweet oranges (8) supports this conclusion. Direct Tissue print-ELISA using the MCAs 3DF1 + 3CA5 is thus the most reliable, sensitive and economic procedure for routine detection of CTV.

Positive results obtained by IC-RT-nested PCR that were not confirmed by indexing may have been due to contaminations in spite of the low risk when a single closed tube is used; alternatively, CTV may in fact have been detected in the samples but was unable to cause infection. In addition, two Mexican limes inoculated with Tissue print ELISA-positive material, gave negative indexing results (no typical symptoms). It is well known that some CTV isolates are unable to induce symptoms in Mexican lime (1).

To summarize, official methods for detection of CTV will include, in addition to biological indexing, ELISA using tissue extracts (DAS-ELISA) or prints on nitrocellulose (Tissue print-ELISA) and/or molecular procedures. Commercial kits using polyclonal antibodies or the

MCAs 3DF1 and 3CA5 are recommended for conventional ELISA, but only the MCAs for Tissue print-ELISA. A combination of two different methods based on biological indexing, serological or molecular detection (with the validated protocols and reagents) will be required to officially underwrite a positive CTV detection.

ACKNOWLEDGMENTS

Financial support was provided by the European Commission, through the SMT program project SMT4-CT98-2252 (DIAGPRO) and by grants from INIA (SC 98-060) and Consellería de Agricultura, Pesca y Alimentación de la Generalidad Valenciana (IVIA grant 5901).

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