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Routine Detection of Citrus Tristeza Virus by Direct Immunoprinting-ELISA Method Using Specific Monoclonal and Recombinant Antibodies

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ABSTRACT. Extract preparation is the most limiting factor for large-scale plant virus testing. Direct tissue print of fresh cross sections of tender shoots or leaf petioles on cellulose membranes, allows the collection of samples and testing of a large number of plants (1,250 plants per team of two workers per day). The printed membranes can be analyzed in the field, mailed, or kept for several months before testing. The analysis is performed by a simple and fast (3 h) direct ELISA protocol using a mixture of citrus tristeza virus (CTV)-specific, alkaline phosphatase conjugated monoclonal antibodies 3DF1 and 3CA5 or using a mixture of 3DF1 and 3CA5 scFv-AP/S recombinant antibodies expressed in *E. coli* as a fusion protein with the alkaline phosphatase enzyme. The sensitivity of immunoprinting-ELISA method was the same as immunocapture-PCR but it was more reliable. A kit has been designed and evaluated under nursery conditions. This kit has been successfully used by nurserymen to test more than 600,000 plants over the last five years.

Index words. citrus tristeza virus, detection kit, immunoprinting-ELISA, tissue print-ELISA, monoclonal antibodies, recombinant antibodies, IC-PCR.

Testing for citrus tristeza virus (CTV) has been performed for many years by grafting on the biological indicator, Mexican lime (16). The application of DAS-ELISA (1, 6) revolutionized the diagnosis by allowing the process of large number of samples in a short period. The ELISA was used for large-scale surveys, CTV control in citrus nurseries, epidemiological, and other studies (4, 8). The ELISA technique has provided diagnosis with a high level of sensitivity and low cost. In addition, the production of monoclonal antibodies (MCAs) specific to CTV in 1982 (14, 15) and its commercialization by Ingenasa (Madrid) made it possible to test a large number of samples for CTV with high sensitivity and specificity. Among the available MCAs (12), the mixture of the well-characterized 3DF1 and 3CA5 is able to recognize all CTV isolates tested from different collections (5). This mixture has been extensively used in routine tests and in several ELISA variants (4). The change in CTV diagnosis due to ELISA with MCAs is illustrated by the fact that

more than 2 million samples have been tested using these MCAs.

The most important limitation for detection of CTV by conventional ELISA, however, is the necessity to prepare plant extracts which is a laborious and time-consuming process especially in woody plants and also enhances risk of contamination. Furthermore, conventional ELISA requires skilled specialists and expensive equipment for reading the plates. A laboratory is necessary for sample extract preparation and testing, and additionally, samples collected for ELISA can only be stored for a maximum of 1 wk at 4°C, before the extract preparation.

The use of membranes to capture and immobilize targets constitutes a good alternative to extract preparation. The development of direct tissue blot immunoassay, immunoprinting-ELISA or tissue print-ELISA in plant pathology (11) and its application to CTV (2, 3, 9), again revolutionized and simplified the detection of the virus. Immunoprinting-ELISA allows the sensitive analysis of thousands of samples in an easy

way, without the need of extract preparation, by directly printing sections of plant material on nitrocellulose membranes.

A complete kit, with improved protocol for CTV detection has been produced by Plant Print Diagnostics (Valencia) in agreement with Instituto Valenciano de Investigaciones Agrarias (IVIA). With this kit more than 600,000 plants have been analyzed since 1994 by nurserymen to test their multiplication blocks, apart from the official control. In this paper, we have evaluated this methodology for its sensitivity, simplicity, cost, as well as the possibility of its use by non-specialized workers, and direct field application under nursery conditions. We also have compared the routine use of conventional MCAs (3DF1 + 3CA5) conjugated to alkaline phosphatase against the use of CTV specific recombinant single chains (scFv-3DF1 and scFv-3CA5) genetically fused to alkaline phosphatase (scFv-AP/s), for the direct detection of CTV by tissue print-ELISA.

MATERIAL AND METHODS

Direct immunoprinting-ELISA protocol. The improved protocol for direct immunoprinting-ELISA was performed in three steps: a) Sample imprinting on nitrocellulose membranes; b) Blocking and addition of CTV-specific antibodies alkaline phosphatase conjugate and; c) Substrate addition and reading. The protocol is performed as follows:

1. **Preparation of plant samples (membrane printing).** Make clean cuts on tender shoots, leaf petioles or fruit peduncles. Press carefully the freshly made sections against the nitrocellulose membrane 0.45 mm (Millipore). Let the trace or the print dry for a few minutes. Printed membranes can be kept for several years in a dry place. For adult plants select five tender shoots (from last flush) or 10 leaves from around the canopy (preferably from the top area) for sample imprint. For seedlings select two shoots or four leaves and analyze. Perform two printings per shoot or one per leaf.
2. **Membrane blocking.** Prepare 1% solution of bovine serum albumin (BSA) in distilled water. Place the membranes (about 7 × 13 cm) in an appropriate container (tray, hermetic container, plastic bag). Pour the BSA solution over the membrane covering them, and incubate for 1 h at room temperature, or overnight at 4°C. A slight agitation is recommended during this step. Discard the albumin solution and keep the membranes in the same container.
3. **Addition of monoclonal antibodies/alkaline phosphatase (AP) linked or recombinant antibodies AP/S fused.** Prepare a solution of CTV specific 3DF1 + 3CA5 MCAs linked to AP (about 0.1 µg/ml each MCA in PBS) or of 3DF1 scFv-AP/S + 3CA5 scFv-AP/S fusion proteins expressed in *E. coli* ¼ diluted in PBS. Pour the solution on the membranes, covering them and incubate for 2 to 3h at room temperature, then discard the conjugate solution.
4. **Washing of membranes.** Prepare 1 l washing buffer (PBS + 0.05% Tween 20) for 10 to 15 membranes each of 7 × 13 cm. Rinse the membranes and the container with 100ml of washing buffer. Wash by shaking (manually or mechanically) with 400 ml buffer for 5 min. Discard the washing buffer and repeat the process with the remaining buffer.
5. **Membrane development.** Prepare substrate buffer by dissolving 10 BCIP-NBT, Sigma Fast tablets in 100 ml (for 10 to 15 membranes) distilled water. Pour

over the membranes and let incubate until appearance of purple-violet color in positive controls (3 to 7 min). Stop the reaction by washing the membranes with tap water. Spread the membranes on absorbent paper and let them dry.

6. **Membranes reading.** Observe the printings by using a low power magnification ($\times 10$ to $\times 20$). Presence of purple-violet precipitates in the vascular region of plant material, reveals the presence of CTV.

Evaluation in citrus nurseries. A complete kit (Plant Print Diagnostics) based on the above described protocol including all reagents and pre-printed controls, was evaluated, in two Spanish nurseries. In Viveros Valencia (Peñíscola, Spain), the method was tested on samples from increase blocks in open field. In Viveros Alcanar (Alcanar, Spain) the samples were collected from an insect-proof screenhouse tunnel. Two-person teams performed the routine analysis of nursery plants. The total number of samples collected daily and printed on membranes was evaluated and compared with the number of extracts prepared from the same samples and analyzed by DAS-ELISA and immunocapture-PCR (IC-PCR). The economic cost of the analysis per nursery plant was also calculated for all assayed techniques.

Comparison with other detection techniques. Samples (five young shoots/tree) from 65 sweet orange trees cv. Washington Navel were analyzed in October by immunoprinting-ELISA. An extract of the same plant material was also analyzed by IC-PCR (7). All these trees have been assayed by DAS-ELISA (15) a year before and proved to be CTV-free. The trees that gave differential diagnostic by both techniques were analyzed again 1 mo later (November) by immunoprinting-ELISA and IC-PCR. Samples were collected again from trees that

gave different test results and analyzed by immunoprinting-ELISA and nested-PCR in single closed tube (13) and grafted on Mexican lime seedlings. In addition 200 seedlings (about 25 cm high) of Mexican lime, Alemow, sour orange and sweet orange (total of 800 plants) were cultivated in containers in field from May to July at Moncada in a clementine plot with 85% CTV infection. The seedlings were individually analyzed by DAS-ELISA biotin/streptavidin system (Ingenasa), tissue print-ELISA and IC-PCR after 6 mo of growth in an insect proof screenhouse.

Production and use of CTV-specific recombinant single chain Fv fragments (scFvs) fused with alkaline phosphatase. The variable domains of 3CA5 and 3DF1 antibody genes were amplified from mRNA isolated from MCAs-producing hybridoma cells kept at Ingenasa (Madrid) and cloned into pDAP2/S (10) vector. The expression of this construct in *E. coli* produced a single chain Fv fragment, in which the two variable domains are connected by a genetically encoded linker, fused to alkaline phosphatase protein. A mixture of both recombinant conjugates 3DF1 scFv-AP/S and 3CA5 scFv-AP/S were assayed by tissue print-ELISA. Parallel assays were performed with conventional MCAs conjugated with alkaline phosphatase.

RESULTS

The collection of nursery plant samples and their direct printing in screenhouse or field allowed the analysis of 1,250 plants/day by a two-person team. About the same number of plants were analyzed daily collecting leaf samples in the field, printing and subsequent analysis in the laboratory conditions (Figs. 1 to 5).

Leaf petioles were the most convenient analysis for tissue print-ELISA in both nurseries. This ma-



Fig. 1. A two person-team collecting samples in open field. Leaves from a plant are held on a wire and each set is separated from another by a piece of plastic. Petioles are cut and membranes imprinted with them in the laboratory of Viveros Valencia (Peñíscola, Spain) citrus nursery.

terial remains succulent over the growth of the plants in the nursery, and can be easily collected without damaging the plants. In addition, prints from leaf petioles occupy less space on a membrane than sections of the stems, allowing a higher number of tests per membrane. Table 1 summarizes the evaluation of the routine analysis of nursery plants performed by a two-worker team by different techniques.

The results of the analysis by DAS-ELISA, tissue print-ELISA and IC-PCR of citrus seedlings exposed to natural CTV infection in Moncada, were coincident. Only five plants out of 800 were detected as CTV in-

fectected by all the assayed techniques. Nevertheless, discrepancies were observed in the analysis of mature sweet orange trees. The comparative analysis by tissue print-ELISA and IC-PCR of 65 recently CTV-infected Washington Navel trees resulted in 37 positive and 16 negative trees by both techniques assayed, five trees tested positive by tissue print-ELISA but negative by IC-PCR, and 7 trees were positive only by IC-PCR. Coincident results were obtained in 81.5% of the analyzed trees but a different diagnosis was rendered in 12 trees. These 12 questionable trees were analyzed again 2 mo later. The five trees that were positive by tis-

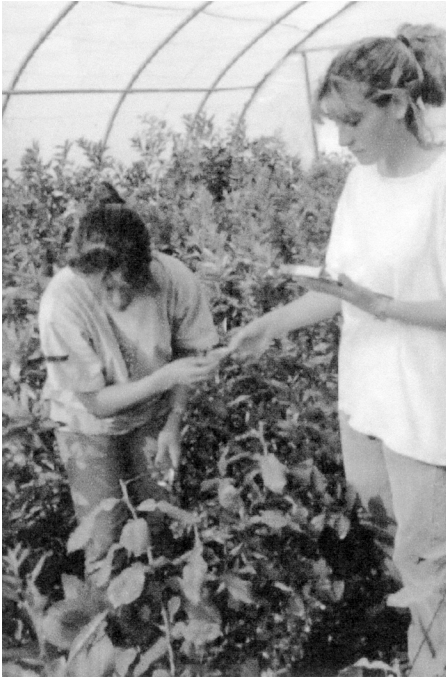


Fig. 2. The first worker collects leaf samples, cuts the petiole and passes each leaf to a second worker who imprints the membrane. The operation is performed under an insect-proof plastic tunnel at Viveros Alcanar (Alcanar, Spain citrus nursery).

sue print-ELISA were confirmed as infected by both techniques. Of the seven trees which were positive by PCR only four were positive by IC-PCR and negative by tissue print-ELISA, and the remaining three



Fig. 3. The same team as in Fig. 2, is able to collect, print and analyze samples from about 1,250 plants per day.



Fig. 4. Overview of a 7 × 13 cm membrane printed with stem sections. The stained vascular area of stem sections indicate that the plants are infected with CTV.

were CTV negative by both techniques. The four trees CTV positive only by PCR were sampled again and analyzed by nested-PCR in a single closed tube and indexed on Mexican lime indicator plant, and all were found negative by both methods.

Printed sections of leaves and stems from CTV infected sweet orange and clementine trees were analyzed 1 and 2 yr after preparation. No differences were observed in this comparison with other samples fresh-printed and subsequently analyzed on the same membrane.

The recombinant antibodies were used to detect CTV coat protein by tissue print-ELISA and the results

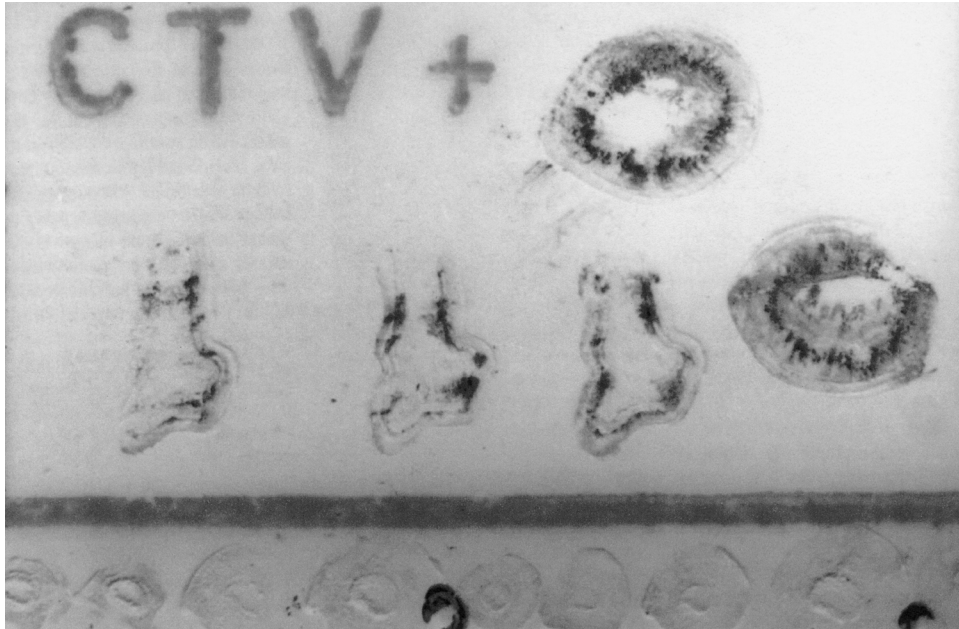


Fig. 5. Stem sections (approximately 10× magnification) from CTV infected plants are used as pre-printed positive controls in commercial kits.

compared with the commercial detection kit (Plant Print Diagnostics) based on the same technique but using the conventional MCAs 3DF1 and 3CA5. The developed prints show a similar intensity and number of stained areas in the vascular region of sections of sweet orange shoots when using conventional MCAs or recombinant antibodies (data not shown).

DISCUSSION

Direct immunoprinting-ELISA or direct tissue print-ELISA performed with universal CTV-specific MCAs is a reliable, sensitive and economic procedure for routine detection of CTV in citrus plants. The sensitivity of the test, using the proposed protocol and tested reagents, is the same as conventional DAS-ELISA or IC-PCR. The advantages of the evaluated tissue print-ELISA kit were their increased reliability compared to IC-PCR, simplicity (that allows its use for non-specialized workers), efficiency (allows to collect and process

up to 1,250 nursery plants per day) and the low cost (\$U.S. 0.26/plant including sampling). In addition, the immunoprinting-ELISA method allows CTV detection in the field or greenhouse in only 3 h after imprint preparation.

The use of immobilized targets on membranes by tissue print or squash, constitute a good alternative to extract preparation. The printed membranes can be stored at room temperature or at 4°C for long periods. Tests performed 2 yr after imprint of samples on a membrane were still producing reliable results. This fact represents a great advantage allowing the storage at room temperature and the submission of pre-printed membranes by conventional courier, if necessary.

The use of tissue print-ELISA kits is very convenient for large surveys in field or nursery plants, and especially more convenient to work in remote sites.

The use of scFv-AP/S fusion proteins which are CTV specific have also proven to be excellent conjugates

TABLE 1
EVALUATION OF THREE DETECTION METHODS FOR THE ROUTINE ANALYSIS OF CITRUS
NURSERY PLANTS BY A TWO-WORKER TEAM

Category	CTV Diagnostic methods ^a		
	DAS-ELISA (extracts)	Immunoprinting-ELISA (tissue prints)	Immunocapture-PCR (extracts)
Sensitivity	+++ ^b	+++	+++
Reliability	+++	+++	++
Simplicity	++	+++	+
Capacity for routine processing	+	+++	+
Number of analyzed plants/day	600	1,250	150
Sampling cost per plant	0.09	0.09	0.09
Extract preparation cost per plant	0.10	0.00	0.22
Reagents, buffers and material, cost per plant	0.50	0.17	1.20
Total cost of the analysis per plant	0.69	0.26	1.51
Laboratory equipment necessary (cost)	15,625	1,250	12,500

^aCost in U.S. \$.

^bThe number of + symbols indicates how methods rate from acceptable (+) to optimum (+++).

in immunoprinting-ELISA. Recombinant conjugates efficiently reacts against CTV and may substitute conventional antibodies linked with alkaline phosphatase by glutaraldehyde in a near future because of its easier production and lower price.

Our experiments in the field clearly showed a higher reliability of the tests performed by immunoprinting-ELISA than by IC-PCR in spite of IC-PCR great theoretical sensitivity. Tissue print-ELISA technique does not have contamination risks or inhibition problems due to plant material extracts.

The tissue print-ELISA method can be easily adopted and adapted to particular conditions in private nurseries. The availability of the evaluated kit makes possible the official Spanish policy to produce only pathogen-free citrus plants. Zero tolerance for CTV in nurseries, in spite of the fact that plants will be infected by viruliferous aphids in the field, ensures that at least some time exists for the safe use of susceptible rootstocks such as Alemow

and that no CTV isolate will be spread from the nurseries (including severe CTV isolates that could be introduced into nursery plantings). The high number of tests performed by private nurserymen, in addition to the official control, also demonstrate the suitability of the method.

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