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Detection of *Citrus psorosis virus* by ELISA, molecular hybridization, RT-PCR and immunosorbent electron microscopy and its association with citrus psorosis disease

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Abstract

Psorosis is a citrus disease of undemonstrated etiology that can be diagnosed by biological indexing on sweet orange seedlings followed by a cross protection test. Its presumed causal agent is *Citrus psorosis virus* (CPsV), type species of the genus Ophiovirus. We compared detection of CPsV by ELISA, RT-PCR, molecular hybridization and immunosorbent electron microscopy, and examined its association with psorosis disease in 11 biologically characterized isolates and in 47 uncharacterized field sources by observation of field symptoms and by biological indexing including the cross protection test. Detection of CPsV by any of the four procedures always coincided with diagnosis of psorosis by cross protection, but it did not always correlate with observation of symptoms thought to be specific, in field trees or in graft-inoculated indicator plants. Trials to detect CPsV by ELISA, molecular hybridization and RT-PCR in citrus sources from different geographical origins, presumed to be psorosis-infected on the basis of field symptoms or reaction of indicator plants, were sometimes unsuccessful, indicating that psorosis symptoms may be induced by causes other than CPsV.

Abbreviations: CPsV – *Citrus psorosis virus*; DIG-probe – digoxigenin-labeled cDNA probe; DTBIA – direct tissue blot immunoassay; ISEM – immunosorbent electron microscopy.

Introduction

Psorosis is widespread and causes serious damage in many citrus growing regions including North and South America and the Mediterranean basin (Roistacher, 1993). The disease is mainly transmitted by propagation of infected buds, but natural spread by unknown means has been reported in some citrus areas of South America (Beñatena and Portillo, 1984). The most characteristic symptoms are bark scaling in the trunk and main branches and internal staining in the underlying wood (Roistacher, 1993). In these areas xylem vessels can be occluded with gum, with decline of the affected branches and reduced yield. Other symptoms including chlorotic flecking or spotting in young leaves, and ringspots in leaves or fruits are sometimes observed (Fawcett, 1932, 1933; Fawcett and Klotz, 1938; Navas-Castillo and Moreno, 1993).

Two types of psorosis have been described, the more common psorosis A, characterized by the

presence of bark scaling only in the trunk and limbs, and the more aggressive psorosis B, causing rampant bark scaling even in fine twigs, chlorotic blotching in old leaves with gummy pustules in the leaf underside, and sometimes ringspots on fruits (Fawcett and Klotz, 1938; Klotz and Fawcett, 1941; Fawcett and Bitancourt, 1943). Wallace and Drake (1968) described citrus ringspot as a different bark-scaling disease inducing yellow blotches, vein banding and/or distinct rings in mature leaves of several indicator species, but later evidence suggested that ringspot and psorosis are variants of the same disease (Derrick et al., 1991; Navas-Castillo and Moreno, 1993; García et al., 1994).

Several other disorders of genetic or infectious origin can cause psorosis-like bark scaling on citrus, particularly, leprosis, Bahía bark scaling, and a bark scaling disorder recently described in Spain (Roistacher 1993; Barbosa et al., 1999; Duran-Vila and Moreno, 2000; Martín et al., 2002b). Furthermore, chlorotic leaf flecking and spotting and ringspots in fruits are also associated with other graft-transmissible but still uncharacterized diseases like concave gum, impietratura, cristacortis or yellow ringspot (Navas-Castillo and Moreno, 1993; Roistacher, 1993; Duran-Vila and Moreno, 2000; Timmer et al., 2000). Hence, diagnosis of psorosis is unreliable when based on field symptoms.

Psorosis is currently diagnosed by biological indexing on sensitive indicator plants, usually sweet orange seedlings (*Citrus sinensis*) grown in a temperature-controlled (18–26 °C) greenhouse (Roistacher, 1991, 1993). Characteristic symptoms include a shock reaction with leaf fall and shoot necrosis in the first flush and/or chlorotic leaf flecking and spotting in the following flushes. Since the latter symptoms are also induced by other graft-transmissible diseases, specific diagnosis of psorosis additionally requires a cross protection test using psorosis B (Wallace, 1957; Roistacher, 1991, 1993; Duran-Vila and Moreno, 2000). In this test, sweet orange seedlings preinoculated with the candidate psorosis isolate are challenge-inoculated with psorosis B. Plants infected with psorosis A do not develop leaf pustules and twig blisters characteristic of psorosis B. The procedure is slow and costly, requires adequate facilities and trained personnel, and cannot be used for large-scale indexing.

Clearly, quick and reliable diagnosis methods based on detection of the pathogen are urgently needed, particularly in citrus areas where the disease spreads naturally. The following candidate approaches have recently become available.

Particles of *Citrus psorosis virus* (CPsV), type species of the genus *Ophiovirus* (Milne et al., 2000), have been partially purified from several psorosis isolates (Derrick et al., 1988; García et al., 1991; Navas-Castillo et al., 1993), and later associated with additional isolates of different origins by Western-blot detection of the coat protein (CP) using an antiserum to the isolate CPV-4 (formerly CRSV-4) from Florida (da Graça et al., 1991; García et al., 1994; Navas-Castillo and Moreno, 1995). Availability of an improved antiserum (García et al., 1997) and of monoclonal antibodies (MAbs) to the CP (Alioto et al., 1999; Potere et al., 1999), has allowed detection of CPsV by double and triple antibody sandwich (DAS- and TAS-) ELISA, and by direct tissue blotting inmunoassay (DTBIA), in biologically characterized and non-characterized psorosis sources (D'Onghia et al., 1998, 2001; Alioto et al., 2000; Roistacher et al., 2000; Martín et al., 2002a). Variation in the epitopes present in the CP of different CPsV isolates (Djelouah et al., 2000; Alioto et al., 2003) makes it advisable to use a mixture of MAbs (Martín et al., 2002a). Antisera have been used to detect CPsV particles using immunosorbent electron microscopy (ISEM) (Derrick et al., 1988, 1991; Navas-Castillo, 1991; García et al., 1994; Navas-Castillo and Moreno, 1995; Milne et al., 1996).

The CPsV genome consists of at least three single-stranded RNAs of negative polarity. RNAs 1, 2 and 3 of the isolate CPV-4 have been sequenced (Barthe et al., 1998; Sánchez de la Torre et al., 1998, 2002; Naum-Onganía et al., 2003), opening new possibilities for CPsV detection by hybridization or reverse transcription (RT) and PCR amplification (García et al., 1997; Barthe et al., 1998; Legarreta et al., 2000; Martín et al., 2002b; Vaira et al., 2003).

Rapid loss of infectivity of CPsV virion preparations *in vitro* has so far prevented direct demonstration that CPsV causes psorosis. Thus, apart from diagnosis considerations, it remains important to see whether the presence of CPsV detected by various methods is indeed strictly correlated with psorosis disease as defined by field symptoms and biological indexing including cross protection.

This aspect has been neglected in most previous work on CPsV diagnosis.

Here, detection of CPsV has been compared by ELISA, molecular hybridization, RT-PCR and ISEM in biologically characterized isolates and in uncharacterized field sources. We have also tested the correlations between positive results in these tests and presence of psorosis as defined by field symptoms, biological indexing and cross protection against psorosis B.

Materials and methods

Psorosis isolates

The biologically characterized psorosis A (P-126, P-129, P-121 and P-AH), psorosis B (PB-102, PB-143 and PB-108) and ringspot-type (RS-101, RS-108 (formerly RS-SR), RS-ALM and RS-SOR) isolates used in this study are part of a collection kept at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Spain. These isolates are maintained in container-grown Pineapple sweet orange propagated on Carrizo citrange (*C. sinensis* × *P. trifoliata*) plants in an insect-proof screenhouse. Data on their pathogenicity characteristics have been published (Navas-Castillo, 1991; Navas-Castillo and Moreno 1993; Martín et al., 2002b) and are summarized in Table 1.

Samples from 47 field trees of different citrus varieties, presumed to be psorosis-infected (or psorosis-free) on the basis of their symptoms, were collected in several citrus-growing areas in eastern Spain. One of these trees was symptomless, one showed concave gum symptoms, one showed atypical bark scaling with a crater-like appearance (Martín et al., 2002b), and 44 trees showed typical psorosis bark scaling and/or chlorotic flecking or spotting in young leaves. Four young shoots from each tree were used to prepare tissue prints on Nylon and nitrocellulose membranes for tissue-print hybridization and DTBIA, respectively (see below). Young leaves of these shoots were pooled, trimmed and used for DAS- and TAS-ELISA, and to prepare RNA extracts for RT-PCR analysis (see below). The extra tissue was desiccated on silica gel for further confirmatory analysis. Budsticks from each tree were used for biological indexing on Pineapple sweet orange and *C. excelsa* (Wester) seedlings, including the cross protection test, as previously described (Martín et al., 2002a). ISEM was performed as described below on leaf extracts of the graft-inoculated *C. excelsa* plants. Data on biological indexing of these field sources and their reactivity with antibodies to the CPsV CP have been published (Martín et al., 2002a) and are summarized in Table 2.

Fifty one sources from different geographic origins, showing psorosis-like bark scaling and/or leaf or fruit symptoms, were also tested for CPsV infection (Table 3). These sources were analyzed by DTBIA and tissue-print hybridization using membranes imprinted at the origin with young shoot sections, or by ELISA, dot-blot hybridization or RT-PCR, using young leaves from the candidate plant, trimmed and desiccated on silica gel at collection time. Most of these sources (Resc Union and those coded with the prefixes B-, DR- and Ps-) were sampled at the collection of exotic citrus pathogens maintained at the quarantine facilities of the USDA at Beltsville Agricultural Research Center (BARC) in Maryland (Garnsey et al., 1987). Samples Pso2, CRSV-4, CRSV 6B-1, and those coded with the prefix FS-, correspond to Florida sources maintained at the former Horticultural Research Laboratory of the USDA at Orlando, whereas sources named Crutch Field and Terra Ceia, also from Florida, were a gift from G. A. Barthe (University of Florida, CREC, Lake Alfred). Isolate CRSV-4 (later named CPV-4), obtained from a Star Ruby grapefruit illegally introduced from Texas (Garnsey et al., 1976), has been widely characterized and its genome sequenced (see above). CRSV 6B-1 was obtained from the field isolate CRSV-6 after passage through single local lesions of *Chenopodium quinoa* (Willd.) and *Gomphrena globosa* (L.) and mechanical transmission to grapefruit (*C. paradisi* Macf.) (Garnsey and Timmer, 1988). All Florida sources were sampled from greenhouse plants. Sources BR 1–6 were from trees affected by Bahía bark scaling (Barbosa et al., 1999) from the research station EMBRAPA-Mandioca e Fruticultura, Bahía (Brazil), kindly provided by C. Barbosa. Sources FPol 1–13, kindly shipped by M. Grisoni (CIRAD, Montpellier, France), correspond to citrus trees of different varieties showing psorosis-like bark scaling in Tahiti and other islands of French Polynesia. Finally, isolates of *Citrus mosaic virus* (B-175), *Citrus tristeza virus* (B-366) and Citrus chlorotic dwarf virus (Kersting

Table 1. Pathogenicity characteristics of several well characterized psorosis A (P-), psorosis B (PB-) and ringspot-type (RS-) isolates and detection of *Citrus psorosis virus* (CPsV) by ELISA, RT-PCR, molecular hybridization and immunosorbent electron microscopy (ISEM)

Isolate	Field symptoms	Symptoms in sweet orange indicator plants	Cross protection	TAS-ELISA[1]	RT-PCR	Molecular hybridization	ISEM
P-126	BS	S, LS	+	+	+	+	+
P-129	BS	S, LS	+	+	+	+	+
P-121	BS	S, LS, NE	+	+	+	+	+
P-AH	BS	S, LS	+	+	+	+	+
PB-102	BS, PsB	S, RS, PsB	NA	+	+	+	+
PB-108	BS, PsB	S, LS, PsB	NA	+	+	+	+
PB-143	BS, PsB	S, LS, PsB	NA	+	+	+	+
RS-101	LS	LS	−	−	−	−	−
RS-ALM	RS, RSF, LS	LS	−	−	−	−	−
RS-108	BS, S, LS, RS, RSF	S, LS, NE	+	+	+	+	+
RS-SOR	BS, LS, DRSF	S, LS, NE	+	+	+	+	+

BS: bark scaling; PsB: blisters in twigs, yellow blotches in old leaves with gummy pustules in the leaf underside; LS: chlorotic flecking or spotting in young leaves; RS: ringspots in leaves; RSF: ringspots in fruits; S: shock reaction (leaf shedding and necrosis of young shoots); DRSF: depressed rings in fruits; NE: necrotic etching. + : positive reaction; − : negative reaction; NA: not applicable.
[1]TAS-ELISA performed with MAb 13C5.

et al., 1996), also from the BARC collection, were included as negative controls.

ELISA

Antibodies. Antiserum A322, an alkaline phospatase (AP) conjugate of its immunoglobulins, and MAbs 13C5 (IgG) and 2A3 (IgM) to the CPsV CP have been described (García et al., 1997; Alioto et al., 1999). For indirect ELISA, rabbit anti-mouse IgG (whole molecule) and goat anti-mouse IgM (μ-chain-specific) immunoglobulins conjugated with AP (Sigma Chemicals) were used in combination with Mabs 13C5 and 2A3, respectively.

DAS- and TAS-ELISA. Plant extracts were prepared by trimming tissue (0.2 g) and blending it in 10 volumes of PBS containing 0.1% Tween 20, 2% (wv^{-1}) polyvinyl pyrrolidone (PVP-10000) and 2.5% ($w\,v^{-1}$) defatted milk powder, using a Polytron homogenizer (Kinematica, Littau, Switzer-

Table 2. Response patterns of different uncharacterized field sources to diagnosis tests for psorosis and for *Citrus psorosis virus* (CPsV)

Diagnostic procedure	Response pattern				
	1	2	3	4	5
Field symptoms (bark scaling)	+	−	+	−	−
Biological indexing (shock and/or young leaf symptoms)	+	+	+	+	−
Cross protection against psorosis B	+	+	−	−	−
Serology (DTBIA[1])	+	+	−	−	−
Serology (DAS-, TAS-ELISA[1])	+	+	−	−	−
Virion detection (ISEM[1])	+	+	−	−	−
Detection of viral RNA (RT-PCR)	+	+	−	−	−
Detection of viral RNA (Molecular hybridization)	+	+	−	−	−
No. of trees showing each response pattern	41	2	1	1	2

[1]DTBIA, TAS ELISA and ISEM performed with MAb 13C5 (Alioto et al., 1999).

Table 3. Pathogenicity characteristics and detection of CPsV in psorosis-like sources of different geographic origin

Source[1]	Origin	Field symptoms	Biological indexing	DTBIA or DAS-/ TAS-ELISA[2]	RT-PCR	Molecular hybridization
B-75	Argentina	NA	LS	+	+	+
B-36	Spain	NA	S, LS	+	+	+
B-88	Spain	LS	S, LS, MT+	+	+	+
B-89	Spain	BS, PsB	S, PsB, LS, MT+	+	+	+
B-96	Spain	BS	S, LS, MT+	+	+	+
B-360	Corsica	NA	LS	+	+	+
CRSV 6B-1	Florida	BS	S, LS, PsB, MT+	+	+	+
Crutch field	Florida	BS	NA	+	+	+
Terra Ceia	Florida	BS	S, LS, PsB, MT+	+	+	+
FS 402	Florida	BS	S, LS, MT+	+	+	+
CPV-4	Florida	BS, RS	S, LS, MT+	+	+	+
FS 386-P	Florida	BS	LS, MT-	–	–	–
FS 281-P	Florida	BS	LS, VB, MT-	–	–	–
FS 385	Florida	BS	LS	–	–	–
Pso2	Florida	LS	NA	–	–	–
B-168	India	BS, LS	LS, VN	–	–	–
B-169	India	NA	LS, VN, VB	–	–	–
B-171	India	BS	LS, VN, VB	–	–	–
B-172	India	NA	LS, VN, VB	–	–	–
B-173	India	BS	LS, VB	–	–	–
B-174	India	BS, LS	NA	–	–	–
B-196	India	NA	LS	–	–	–
BR 1-6	Brazil	BS	NA	NA	–	–
FPol 1-13	French Polynesia	BS	NA	–	–	NA
DR 98-94	Dominican Rep.	BS	NA	–	–	–
DR 96-116	Dominican Rep.	BS	NA	–	–	–
DR-96-117	Dominican Rep.	BS	NA	–	–	–
DR 96-119	Dominican Rep.	BS	NA	–	–	–
DR 96-121	Dominican Rep.	BS	NA	–	–	–
DR 96-122	Dominican Rep.	BS	NA	–	–	–
DR 96-123	Dominican Rep.	BS	NA	–	–	–
Ps1	R. South Africa	RSF	NA	–	–	–
Ps 2	R. South Africa	RSF	NA	–	–	–
Resc. Union	R. South Africa	RSF	NA	–	–	–

BS: bark scaling; LS: chlorotic flecking or spotting in young leaves; RS: ringspots in leaves; RSF: ringspots in fruits; S: shock reaction (leaf shedding and necrosis of young shoots); PsB: blisters in twigs, yellow blotches in old leaves with gummy pustules in the leaf underside; VB: vein banding; VN: vein netting; MT+: mechanically transmitted to *C. quinoa*; MT–: mechanical transmission to *C. quinoa* failed; NA: not done or data not available.

[1]BR 1–6: six individual trees from Bahía (Brazil); FPol 1–13: 13 individual trees from French Polynesia.

[2]DAS-ELISA performed with antiserum A322 and DTBIA and TAS-ELISA with MAbs 13C5 and 2A3.

land). Plate coating, antibody incubations and washing conditions for DAS- and TAS-ELISA were performed as described by Alioto et al. (1999), except that plates for DAS-ELISA were coated using a 1/4000 dilution of antiserum A322. Optical density (OD) at 405 nm was measured in a Titertek Multiscan® Plus (Laboratory Systems, Helsinki, Finland). Each sample was analyzed in at least two wells, and six wells with healthy plant extract were included in each plate as negative controls. Mean experimental readings at least three times the mean reading of the negative controls were considered positive.

Direct tissue blotting immunoassay (DTBIA). DTBIA of tender young shoots was performed as described in Martin et al. (2002a).

RT-PCR

Detection of CPsV by RT-PCR was performed using total RNA extracts as template and primers

Ps66 (5′-TCGAAGCTGTATGATGGTGA, positions 768–787 on RNA3) and Ps65 (5′-TGCCATCTGGAGTGAGGCT, positions 1182–1200 on RNA3). The sequence of these primers was based on regions of the CP gene highly conserved in 23 psorosis isolates from different geographic areas (unpublished data).

Total RNA was extracted from 100 mg of fresh tissue (or 20 mg of tissue desiccated on silica gel) with phenol/guanidine isothiocyanate (TRIZOL®, Life Technologies), following the manufacturer's instructions for samples with high sugar content, but extending the extraction period of desiccated samples to 30 min. RNA was resuspended in 50 µl of DEPC-treated distilled water.

One-step RT-PCR was conducted in a 25-µl reaction mixture containing 1 µl of total RNA extract, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM of each dNTP, 1 µM of each primer, 20 U of SuperScript II reverse transcriptase and 1 U of *Taq* DNA polymerase (GIBCO BRL). The thermocycling conditions included 45 min at 42 °C for RT, 2 min at 94 °C for inactivation of reverse transcriptase and initial denaturation, 40 cycles of 15 s at 94 °C, 15 s at 45 °C and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products (5 µl) were analyzed in a 2% ($w\,v^{-1}$) agarose minigel using 1×TAE (40 mM Tris-acetate pH 8.3, 1 mM EDTA) as electrophoresis buffer and 100 V for 1 h. Gels were stained in 0.5 $\mu g\,ml^{-1}$ ethidium bromide and observed on a UV-transilluminator. The 1-Kb plus ladder (Invitrogen) was used for size estimation of the PCR products.

Molecular hybridization

To prepare a CPsV-specific probe, cDNA of the 3′ proximal half of the CP gene was obtained by RT-PCR in the conditions previously described, using primers CPV-1 and CPV-2 (Barthe et al., 1998) and total RNA extract from citrus tissue infected with CPsV isolate P-121. After cloning in the pGem-T plasmid vector (Sambrook et al., 1989), cDNA was digoxigenin labeled using the same primers and the PCR DIG-labeling and detection kit (Roche). For dot-blot hybridization, total RNA extracts were denatured at 94 °C for 5 min in 50% formamide, chilled on ice, and then 1 µl of denatured RNA was spotted onto Nylon membranes (Hybond-N, Amersham Pharmacia Biotech). For tissue-print hybridization, imprints were prepared by transversally cutting tender shoots and gently pressing the fresh cuts on Nylon membranes (Garnsey et al., 1993). After air drying and UV cross-linking (50 mJ), the membranes were prehybridized in 0.02% ($w\,v^{-1}$) SDS, 50% formamide, 5×SSC (750 mM NaCl, 75 mM sodium citrate, pH 7), 2% ($w\,v^{-1}$) blocking reagent (Roche) and 0.1% ($w\,v^{-1}$) lauryl sarcosine, for 1 h at 42 °C, hybridized for 16 h at 42 °C in the same solution with the DIG-probe, and then washed twice (5 min each) in 2× SSC, 0.1% ($w\,v^{-1}$) SDS at room temperature, and twice (15 min each) in 0.5× SSC, 0.1% ($w\,v^{-1}$) SDS at 42 °C. Hybridization was detected with the DIG Nucleic Acid Detection kit (Roche) according to the manufacturer instructions, except for two additional washes with maleate buffer and 0.3% Tween 20 performed after incubation with the alkaline phosphatase-conjugated anti-DIG antibody (Narváez et al., 2000). The reaction was revealed using chemiluminescent substrates CSPD or CDP-star (Roche) and exposing the film for 2 h at 37 °C (CSPD) or for 30 min at room temperature (CDP-star).

Immunosorbent electron microscopy (ISEM)

The general procedure was as described in Milne (1993). Extracts were prepared by homogenizing about 6 mm^2 leaf tissue from single leaves in 50 µl of 0.1 M phosphate buffer, pH 7, containing 2% ($w\,v^{-1}$) PVP-44000 (BioRad). Carbon-Formvar coated grids were floated for 30 min on a drop of a 1/1000 dilution of ascites containing MAb 13C5 (Alioto et al., 1999) in 0.1 M phosphate buffer, pH 7, rinsed with phosphate buffer, drained, and then floated on the plant extract for 2 h at room temperature or at 4 °C overnight. After rinsing with distilled water, the grids were stained with 1% uranyl acetate, dried, and examined in a Philips CM 10 EM at 60 KV. Grids were examined at 25,000× instrument magnification using a 10× binocular. A positive result (generally 10–100 virus particles per 400-mesh grid square) was checked using a second sample, while a negative result (no virus particles) was repeated four more times.

Results

Detection of CPsV by laboratory methods in biologically characterized sources

Detection of CPsV by ELISA, RT-PCR, molecular hybridization and ISEM were compared by analyzing a group of biologically characterized psorosis A, psorosis B and ringspot-type isolates of the IVIA collection (Table 1). CPsV was detected by the four procedures in all isolates of psorosis A and B, and in two of the ringspot-type isolates (RS-108 and RS-SOR), but not in the other two (RS-101 and RS-ALM). Classification of RS-101 and RS-ALM as ringspot was based on the presence of this specific symptom on leaves and/or fruits (Wallace and Drake, 1968; Timmer et al., 1978; Timmer and Garnsey, 1981). However, these isolates neither showed bark scaling in the field nor induced the shock reaction in sweet orange indicator plants, and they did not protect against challenge inoculation with psorosis B (Navas-Castillo and Moreno, 1993; Table 1). Interestingly, CPsV was not detected in these two isolates by any of the four procedures tested.

Similar results were obtained by dot-blot or tissue-print hybridization, or by RT-PCR using fresh or silica-desiccated tissue.

Detection of CPsV in uncharacterized field sources

To further compare reliability of ELISA, RT-PCR, molecular hybridization and ISEM for CPsV detection and to check the association of CPsV with psorosis disease, as diagnosed by field symptoms and by biological indexing, 47 uncharacterized field sources of different varieties were analyzed (Table 2). Again, detection of CPsV in those sources by any of the four procedures was always coincident and correlated with psorosis diagnosis based on the cross protection test. Forty one sources showing typical bark scaling (pattern 1) and two non-scaled trees with young-leaf symptoms (pattern 2) induced shock and/or foliar symptoms in indicator plants, protected against psorosis B, and were CPsV-infected, as detected by the four procedures used. One source showing atypical bark scaling (pattern 3) and one showing concave gum symptoms (pattern 4) induced foliar symptoms in indicator plants, but did not protect against psorosis B and were CPsV-negative by all detection methods. The symptomless tree and one tree showing only chlorotic mottling were symptomless in indicator plants, did not protect against psorosis B and were CPsV-negative by all detection procedures (pattern 5).

CPsV detection in psorosis-like sources of different geographic origins

To assess if CPsV was associated with psorosis-like disorders found in different citrus areas, 51 additional symptomatic sources from nine geographic origins were analyzed by DTBIA or DAS- and TAS-ELISA, RT-PCR and dot-blot or tissue-print hybridization using biologically inactive material. These sources were presumed to be psorosis-infected based on field symptoms (bark scaling or young leaf patterns) and, in a few cases, on symptoms induced on indicator plants, but none of them had been tested for cross protection against psorosis B. Data available on their pathogenicity profiles are summarized in Table 3.

CPsV was detected in 11 of these sources but not in the other 40. Again, sources positive by ELISA, were also positive by RT-PCR and molecular hybridization (ISEM not tested), and those reacting negatively by ELISA also failed to react by the other procedures.

Seven of the 11 CPsV-infected sources were known to cause bark scaling, and for the other four, available records do not show whether or not they would induce bark scaling in appropriate conditions. At least eight of the 11 CPsV-infected sources caused a shock reaction in indicator sweet orange seedlings and seven of them were mechanically transmitted to *C. quinoa* and induced characteristic local lesions (Garnsey and Timmer, 1988). The Crutch field source from Florida, taken from a bark-scaled tree, induced no symptoms on Pineapple sweet orange indicator plants (G.A. Barthe, personal communication).

CPsV was not detected in three sources from Florida, four from India, six from Brazil, thirteen from French Polynesia and seven from the Dominican Republic, whose original field trees showed bark scaling. The same was true for other sources from Florida (one), India (three), and South Africa (three) causing different leaf or fruit patterns in the field or in indicator plants. Finally, CPsV was never detected in extracts from healthy sweet orange seedlings or from tissue infected with

Citrus mosaic virus, *Citrus tristeza virus* or Citrus chlorotic dwarf virus, included as controls.

Discussion

Our results indicate that CPsV can be readily detected, not only by different ELISA formats using antibodies to the CP gene, but also by procedures detecting viral RNA (RT-PCR and dot-blot or tissue-print hybridization) or virions (ISEM), with coincident results. They also indicate that detection of CPsV is closely associated with psorosis diagnosis based on cross protection against psorosis B.

A pending question in citrus pathology is to show if CPsV is the causal agent of psorosis disease, which in turn requires a precise definition of the psorosis syndrome. In this regard, the literature on psorosis identification has become noticeably confused over the years. Since bark scaling was the first and characteristic symptom of the disease, some psorosis reports were based on the simple observation of this symptom without further testing for its transmissibility. After observation that grafts from bark-scaled trees were able to induce young leaf symptoms in sweet orange seedlings (Fawcett, 1932, 1933), graft inoculation of this indicator plant was used for quick psorosis diagnosis (Wallace, 1945), although other graft-transmissible diseases were later shown to induce similar symptoms in the same test (Duran-Vila and Moreno, 2000; Timmer et al., 2000). The discovery that lesion bark inoculum was able to induce psorosis B in sweet orange seedlings, and that only psorosis A-infected seedlings showed cross protection against psorosis B (Fawcett and Klotz, 1938; Fawcett and Bitancourt, 1943; Wallace, 1957), provided a specific diagnosis test (Roistacher, 1991, 1993). Many subsequent reports on psorosis diagnosis failed, however, to use it. For some time, additional confusion was added by the consideration of ringspot-type psorosis as a different disease from psorosis (Wallace and Drake, 1968).

CPsV has been partially purified from several sources but instability of the virions has so far impeded fulfillment of Koch's postulates for psorosis. Although development of antibodies to the CP allowed detection of CPsV in different psorosis-like sources, to our knowledge only one report (Roistacher et al., 2000) presented appropriate data on indexing for psorosis using the cross protection test. Also, detection of CPsV only with MAbs might have missed some CPsV variants lacking the epitope/s recognized by those MAbs (Djelouah et al., 2000; Mrani et al., 2002; Alioto et al., 2003).

In this work, the presence of CPsV in biologically characterized isolates, as detected by ELISA, RT-PCR, molecular hybridization or ISEM, was correlated with psorosis as diagnosed by biological indexing and cross protection. This correlation was observed in isolates of different pathogenicity, regardless of symptoms caused on indicator plants, which ranged from only mild flecking in young leaves to necrotic etching or psorosis B pustules in adult leaves. We conclude that isolates affording no protection against psorosis B were CPsV free, even if they induced chlorotic patterns reminiscent of psorosis in field or indicator plants. This result was confirmed by analysis of 47 uncharacterized field sources. Again, perfect correlation was obtained between the presence of CPsV as detected by ELISA, RT-PCR, molecular hybridization and ISEM, and psorosis diagnosed by cross protection, independently of the presence or absence of bark scaling in field trees or the ability to induce chlorotic patterns in young leaves. For example, a field source with atypical bark scaling, shown to be CPsV-negative by ELISA and biologically different from psorosis (Martín et al., 2002a,b), was confirmed to be CPsV free by the other three procedures.

Our results strongly suggest that CPsV must be the causal agent of psorosis, as defined by the cross protection test against psorosis B, and that rapid diagnosis of the disease can be obtained by one or more of the procedures described here. The new diagnosis methods are more reliable than observation of bark scaling in the field or performing biological indexing without the cross protection test, as frequently done in reports on psorosis incidence.

Analysis by ELISA, RT-PCR and molecular hybridization of sources from different geographic origins, presumed to be psorosis-affected, showed that most of them were CPsV free. Many of these had been collected from bark-scaled field trees and others induced young leaf patterns in indicator plants, but identification of these symptoms as psorosis by the cross protection test could not be

done. Based on symptom observation psorosis has been presumed to be a disease widespread in most citrus areas, however, data presented here suggest that CPsV incidence might be more limited than initially thought and that symptoms reported in some cases might be induced by causes other than CPsV. One source (Crutch field from Florida) from a bark-scaled tree was asymptomatic on sweet orange indicator plants, but proved CPsV-positive, further confirming the limited reliability of symptom observation.

As the results of ELISA, RT-PCR, molecular hybridization and ISEM were always coincident, the procedure of choice on each occasion will depend on the diagnosis purpose and on the facilities available. ISEM may be convenient to quickly examine a few samples, but a high quality and well adjusted electron microscope and an experienced microscopist are usually required to reliably identify CPsV virions (Milne et al., 1996).

DTBIA and tissue-print hybridization may be best for large scale testing in field surveys and epidemiological studies, as no sample processing is required and tissue prints can be prepared in the field. Imprinted membranes can be sent elsewhere for processing, as were membranes imprinted with sources from French Polynesia or from the BARC collection in this work, thus avoiding quarantine risks. Membranes can also be stored for long periods before processing – indeed, some nitrocellulose or Nylon membranes used in this work were processed 2–3 years later by DTBIA or tissue-print hybridization, respectively, with results similar to those obtained with fresh or desiccated tissue. However, reliable CPsV detection by DTBIA requires printing with tender juicy shoots or ovaries (D'Onghia et al., 2001; Martín et al., 2002a).

RT-PCR is usually considered the most sensitive method for viral RNA detection, but it is not appropriate for large scale indexing, and the risk of contaminations or false negatives produced by enzyme inhibitors in citrus extracts may reduce its reliability. Overall, DAS- and TAS-ELISA and dot-blot hybridization are appropriate for most applications and can be safely used with various tissue types, albeit the latter is less reliable with old leaves. However, as in most diagnosis situations, the safest decision is to use more than one detection procedure.

The above methods can contribute to simplify and improve the accuracy of future studies on psorosis but biological indexing on sweet orange seedlings will continue to be necessary to guarantee that citrus budwood obtained through sanitation, quarantine and certification programs (Navarro, 1993) is also free from other graft-transmissible pathogens that induce young leaf symptoms.

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