Plant Pathology (2002) **51**, 134–141

Detection of *Citrus psorosis virus* in field trees by direct tissue blot immunoassay in comparison with ELISA, symptomatology, biological indexing and cross-protection tests

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Serological detection of *Citrus psorosis virus* (CPsV) by direct tissue blot immunoassay (DTBIA) and by double (DAS) and triple (TAS) antibody sandwich ELISA, was compared in samples from various citrus varieties growing in the glasshouse and in the field. In young shoots and leaves, CPsV was readily detected by the three procedures, whereas DTBIA detection in old leaves was less consistent. DTBIA detection and ELISA readings in nine different citrus varieties were similar, suggesting that CPsV accumulates to equivalent levels in all of them. In infected field trees from Spain or Italy, CPsV was consistently detected by TAS ELISA, even in samples of old leaves in winter, whereas DTBIA detection in the same trees was reliable only when using young shoots. Detection of CPsV by DTBIA and by DAS and TAS ELISA in previously untested field trees correlated perfectly with psorosis diagnostics based on biological indexing, specifically with the capacity of those sources to cross-protect against challenge inoculation with psorosis B. Some trees without bark scaling were shown to be psorosis-infected by biological indexing and to contain CPsV by serological tests; other trees showing psorosis-like bark or leaf symptoms in the field were shown to be psorosis-free by biological indexing and also CPsV-free by serology. This is the first time that the presence of CPsV has been correlated with psorosis infection as diagnosed by biological indexing.

Keywords: citrus varieties, CPsV, DTBIA, psorosis A, psorosis B

Introduction

Psorosis is one of the longest-known and most widespread graft-transmissible diseases of citrus (Swingle & Webber, 1896). The most characteristic symptoms of the disease in adult field trees are bark scaling and internal wood staining in the main trunk and limbs (Roistacher, 1993). Chlorotic flecking on young leaves is sometimes observed in the spring flush. Psorosis B (Fawcett, 1932, 1933), a more aggressive form of the disease than the common type, psorosis A, produces extensive bark scaling which also affects secondary branches, chlorotic blotching in old leaves with gummy pustules on the underside, and sometimes ringspot on fruits (Fawcett & Klotz, 1938; Klotz & Fawcett, 1941; Fawcett & Bitancourt, 1943). Psorosis rarely kills the plant, but trees may show thin foliage, twig dieback, stunting, low yield and small-sized fruits, particularly when the B form is present.

Psorosis is primarily spread by propagation of infected buds, but several observations in Argentina, Texas and California suggest that natural spread by an unknown vector may have occurred in some cases (Timmer & Garnsey, 1980; Roistacher, 1993). The fact that bark scaling rarely appears in trees less than 10 years old probably favours propagation of infected buds by growers, who presume them to be pathogen-free. This may partly explain the high disease incidence in some areas.

In most situations, damage can be avoided by propagating certified psorosis-free budwood (Navarro, 1993), and indeed the first certification programme, in California, was based on biological indexing of budwood sources (Fawcett, 1938; Hiltabrand, 1957). Indexing is performed by grafting tissue to sensitive indicator plants, usually young seedlings of sweet orange (*Citrus sinensis*). These plants are then held in a temperature-controlled glasshouse and observed for development of characteristic symptoms (Roistacher, 1991), which include a shock reaction with leaf shedding and dieback of the first flush and/or

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Accepted 2 November 2001.

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chlorotic leaf flecking and spotting in successive flushes. Since other graft-transmissible diseases also induce chlorotic patterns in young leaves, more specific diagnosis requires a cross-protection test using psorosis B as challenge inoculum (Fawcett & Cochran, 1942; Wallace, 1957; Roistacher, 1991, 1993). In this test, healthy sweet orange seedlings inoculated with psorosis B show the characteristic symptoms within 6 months, whereas plants already infected with psorosis A are protected and do not show these symptoms. Psorosis B isolates are less frequent in the field and they can be directly diagnosed on sweet orange seedlings, where they induce characteristic pustules and blisters.

Biological indexing is slow and costly, requires an insect-proof temperature-controlled glasshouse and trained personnel, and cannot be applied for massive field indexing. Clearly, in areas where psorosis may spread naturally or is still being spread through infected budwood, quick and reliable diagnostic procedures are urgently needed for epidemiological and control purposes.

The causal agent of psorosis is presumed to be *Citrus psorosis virus* (CPsV), the type member of the genus *Ophiovirus* (Milne *et al.*, 2000). CPsV virions are highly kinked filaments 3–4 nm in diameter of at least two sizes (García *et al.*, 1994), which contain three single-stranded RNAs of negative polarity and a 48- to 50-kDa coat protein (Sánchez de la Torre *et al.*, 1998; Milne *et al.*, 2000).

After first trials to purify the virions (Derrick *et al.*, 1988; García *et al.*, 1991; Navas-Castillo *et al.*, 1993), polyclonal and monoclonal antibodies (MAbs) to the virus were developed that allowed detection of CPsV by ELISA in citrus extracts (García *et al.*, 1997; D'Onghia *et al.*, 1998; Alioto *et al.*, 1999, 2000; Potere *et al.*, 1999). Some of these papers give information on the incidence of CPsV in different citrus varieties in Italy, but in most cases specific data on the correlation between ELISA reaction and psorosis infection, as detected by the presence of bark scaling and by proper biological indexing, are not provided, and diagnosis based on cross-protection is not considered in any of them.

Direct tissue blot immunoassay (DTBIA) has been used for rapid detection of several viruses (Lin *et al.*, 1990; Hsu & Lawson, 1991; Garnsey *et al.*, 1993; Makkouk & Comeau, 1994; Hsu *et al.*, 1995; Makkouk & Kumari, 1996) and recently, D'Onghia *et al.* (2001) have briefly reported CPsV detection by DTBIA in citrus flowers and ovaries. This paper describes the development and evaluation of DTBIA and ELISA for detection of CPsV and correlation between the presence of CPsV and the presence of psorosis, as defined by field symptoms, indicator plants and cross-protection against psorosis B.

Materials and methods

Virus isolates and hosts

The CPsV isolates P121, P126, PB108 and RS-SR used in this study are part of a collection kept at the Instituto Valenciano de Investigaciones Agrarias (IVIA) and have been biologically characterized. P121 and RS-SR induce severe symptoms in several hosts (Navas-Castillo & Moreno, 1993), whereas P126 induces flecking and mottling in young leaves and occasionally shock in the first flush (S. Martín, unpublished data). PB108 induces psorosis B symptoms and has been used for years as challenge inoculum in cross-protection tests (Navas-Castillo & Moreno, 1993). These isolates are maintained in container-grown sweet orange cv. Pineapple plants in an insect-proof screen house. For this study, they were graft-inoculated (Roistacher, 1991) onto plants of the following varieties: sweet orange cvs Pineapple, Washington navel, Salustiana and Valencia Late, lemon (*C. limon*) cv. Fino, grapefruit (*C. paradisi*) cv. Marsh, clementine (*C. clementina*) cv. Nules, satsuma (*C. unshiu*) cv. Okitsu, mandarin hybrid (*C. clementina* × *C. tangerina*) cv. Fortune and tangor (*C. reticulata* × *C. sinensis*) cv. Ortanique. Pineapple sweet orange plants were seedlings, Fino lemon was propagated on Alemow (*C. macrophylla*) seedling rootstocks and the other varieties were propagated on Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*) seedling rootstocks. These plants were grown in a temperature-controlled glasshouse (18–26°C) using a potting mix of 50% sand and 50% peatmoss and a standard fertilizing procedure (Arregui *et al.*, 1982).

Field samples of different varieties of sweet orange, grapefruit, lemon, clementine, satsuma and sour orange (*C. aurantium*), showing psorosis-like symptoms, or known to be infected with uncharacterized CPsV, were also collected in various citrus growing areas in Spain and Italy. For biological indexing of field sources two seedlings each of Pineapple sweet orange and *C. excelsa* were graft-inoculated with bark pieces from each candidate tree. After symptom evaluation in at least two flushes, Pineapple sweet orange plants were challenge-inoculated with isolate PB108 and evaluated for cross-protection 3 and 6 months later (Roistacher, 1991).

Antibodies

Antiserum A322, a conjugate of antibodies from A322 with alkaline phospatase (AP), and MAbs 13C5 (IgG) and 2A3 (IgM) to CPsV was used as previously described (García *et al.*, 1997; Alioto *et al.*, 1999). For indirect assays, rabbit antimouse IgG (whole molecule) and goat antimouse IgM (μ-chain-specific) immunoglobulins conjugated with AP (Sigma, Madrid, Spain) were used in combination with MAbs 13C5 and 2A3, respectively.

Direct tissue blot immunoassay (DTBIA)

Tissue prints were prepared by transversely cutting tender shoots, petioles or rolled leaf blades and gently pressing the freshly cut surface onto membranes of nitrocellulose of 0·45-μm pore size (Bio-Rad, Madrid, Spain) or nylon (Amersham Pharmacia, Barcelona, Spain, or Roche Diagnostics, Barcelona, Spain). The prints were air-dried and blocked for 30 min in TBS buffer (10 mM Tris-HCl, pH 7·4, 0·15 M NaCl) containing 50 g L^{-1} defatted milk

powder (TBS-milk buffer) or in PBS buffer (8 mM Na_2HPO_4, 1·5 mM KH_2PO_4, 2·7 mM KCl, 0·14 M NaCl, 3 mM NaN_3, pH 7·4) containing 20 g L^{-1} Triton X-100 and 50 g L^{-1} defatted milk powder (PBS-milk buffer). In the latter case, the membranes were washed three times in distilled water before incubation with antibodies. For indirect serological detection, membranes were incubated for 90 min in a 1/10 000–1/50 000 dilution of ascites fluid containing MAb 13C5 or 2A3, or a 1:1 (v:v) mixture of these, in TBS-milk or PBS-milk buffer. After three washes of 15 min each with TBST or PBST (TBS or PBS plus 3 g L^{-1} Tween 20) and two washes (the first and the last) with distilled water, membranes were incubated for 90 min in a 1/20 000 dilution in TBS-milk or PBS-milk buffer with the appropriate AP-conjugated antibody. Membranes were then washed as before and equilibrated in substrate buffer (0·1 M Tris-HCl, pH 9·5) for 5 min before adding the substrate. For direct detection, the membranes were blocked as described, incubated for 3 h in a 1/10 000 dilution of the A322 antibodies conjugated with AP in TBS-milk buffer, and then washed and equilibrated in substrate buffer as before. All incubations were at room temperature. AP activity was detected with the chromogenic substrate BCIP/NBT (Blake *et al.*, 1984) or with the chemiluminiscent substrates CSPD or CPD-star (Roche Diagnostics), according to the manufacturer's instructions.

DAS and TAS ELISA

Plant extracts were prepared as described by Alioto *et al.* (1999) or by trimming tissue (0·2 g) and blending it in 10 volumes of PBS containing 1 mL L^{-1} Tween-20, 20 g L^{-1} polyvinyl pyrrolidone (PVP-10 000) and 25 g L^{-1} defatted milk powder, using a Polytron homogenizer (Kinematica, Littau, Switzerland). DAS ELISA with polyclonal antibodies (A322) was performed as described by García *et al.* (1997). Plate coating, antibody incubations and washing conditions for TAS ELISA were as described by Alioto *et al.* (1999). Optical density (OD) at 405 nm was measured using a Titertek Mutiscan® Plus (Laboratory Systems, Helsinki, Finland) or a Biorad 3550 Microplate reader.

Each sample was tested twice in each plate, together with six wells prepared from healthy citrus leaf (negative controls). Mean experimental readings at least three times the mean of the negative controls were considered positive. In experiments where different citrus varieties and sampling of different tissues in different seasons were compared, six positive-control wells were also included in each plate. They contained 1/50 dilutions of a single pool of desiccated and powdered young leaves from several CPsV-infected plants. The ELISA readings of these controls were within the range 0·300–0·550, and those of the negative controls within the range 0·002–0·013, using MAb 13C5.

To obtain the final ELISA results (Table 1), the mean negative control values for each plate were subtracted from each experimental value and from the mean positive control value. Finally, the adjusted experimental values were expressed as a percentage of the adjusted positive control, allowing readings from different plates to be compared.

Results

CPsV detection by DTBIA in glasshouse-grown plants

To determine the best tissue for detection of CPsV by DTBIA, young shoots, young leaves about half grown, young fully expanded leaves and old hardened leaves from glasshouse-grown sweet orange plants cv. Pineapple, healthy or infected with psorosis isolates RS-SR, P126 or PB108, were sectioned and printed on nitrocellulose membranes. After incubation with antiserum A322, with MAb 13C5 or 2A3, or with a 1:1 (v:v) mix of both MAbs, the reaction was developed with BCIP/NBT or CSPD.

CPsV was readily detected in tissue blots of young shoots and leaves (half or full size) from the CPsV-infected plants, whereas blots from old infected leaves gave weaker signals and detection was less consistent (Fig. 1). No reaction was observed in equivalent prints from healthy plants. Young tender shoots 2–5 mm in diameter were easy to handle, and whenever possible this tissue was used in subsequent tests of candidate trees.

CPsV was detected with similar sensitivity using nitrocellulose or nylon membranes, TBS or PBS in the extraction buffer, and development with either BCIP/NBT or

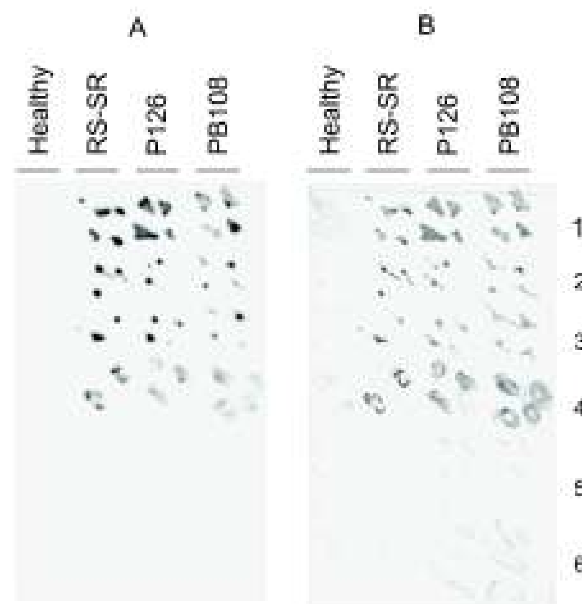

Figure 1 Direct tissue blot immunoassay (DTBIA) detection of *Citrus psorosis virus* (CPsV) in several types of tissue from healthy sweet orange seedlings and those infected with psorosis isolates RS-SR, P126 or PB108. Tissue prints were prepared on a nitrocellulose membrane using young shoots (1), petioles of half-size young leaves (2), petioles (3) or rolled leaf blade (4) of fully expanded young leaves, and petioles (5) or leaf blade (6) of old leaves. The membrane was developed with the chemiluminiscent substrate CSPD (A) and then with the chromogenic substrate BCIP/NBT.

Table 1 Comparison of TAS ELISA[a] and direct tissue blot immunoassay (DTBIA)[b] with monoclonal antibody 13C5 for *Citrus psorosis virus* (CPsV) detection in field trees (already established as CPsV-positive) in Spain and Italy, in different tissues and at different seasons

	Winter		Spring				Summer			
	Old leaves		Young shoots		Old leaves		Young shoots		Old leaves	
Tree[c]	TAS-ELISA	DTBIA	TAS-ELISA	DTBIA	TAS-ELISA	DTBIA	TAS-ELISA	DTBIA	TAS-ELISA	DTBIA
Trees in Spain										
L1	12·9 ± 5·9	0/5	100·1 ± 27·8	6/6	14·6 ± 8·4	0/6	92·2 ± 16·9	5/5	19·7 ± 16·4	2/6
L2	32·9 ± 6·9	3/4	105·5 ± 21·0	6/6	11·6 ± 5·3	0/6	62·4 ± 33·1	6/6	11·4 ± 8·4	0/6
G1	21·4 ± 7·1	2/6	117·4 ± 44·5	6/6	26·4 ± 9·3	0/6	69·2 ± 31·4	5/6	22·6 ± 12·2	2/6
G2	28·2 ± 23·5	1/6	140·3 ± 28·1	6/6	34·2 ± 16·4	0/6	103·7 ± 25·6	4/4	24·4 ± 20·6	0/5
P1	32·1 ± 9·8	1/6	100·5 ± 16·9	6/6	15·4 ± 14·7	0/6	41·7 ± 13·2	0/6	8·9 ± 4·3	0/4
P2	26·8 ± 18·0	1/6	53·9 ± 6·2	6/6	26·6 ± 17·1	0/6	26·7 ± 10·3	0/6	24·0 ± 8·0	0/3
W1	67·8 ± 16·3	1/4	101·2 ± 25·7	5/6	22·1 ± 10·1	0/6	94·2 ± 17·1	4/4	70·2 ± 12·2	0/4
W2	35·9 ± 26·4	1/6	115·3 ± 14·2	6/6	26·4 ± 8·5	0/6	89·2 ± 41·5	2/6	13·2 ± 2·6	0/6
W3	42·1 ± 13·2	1/6	76·7 ± 18·4	6/6	19·7 ± 6·6	0/6	46·3 ± 17·8	6/6	26·4 ± 16·4	3/6
W4	35·0 ± 6·3	4/6	103·8 ± 18·2	6/6	40·9 ± 30·8	0/6	63·7 ± 19·4	3/6	31·9 ± 31·9	2/6
Trees in Italy										
SO	5·1 ± 2·7	0/4	ND	ND	50·5 ± 20·8	4/6	96·0 ± 46·2	5/6	15·4 ± 8·8	1/6
B	14·0 ± 4·4	0/6	198·0 ± 20·9	6/6	72·5 ± 15·6	0/6	61·2 ± 22·3	6/6	24·8 ± 7·0	6/6
T	11·6 ± 3·5	0/6	292·1 ± 35·9	6/6	61·1 ± 14·7	1/6	88·1 ± 38·8	6/6	17·6 ± 11·2	4/6
M1	12·6 ± 4·3	0/6	319·5 ± 79·4	5/6	61·8 ± 25·5	0/6	60·2 ± 18·5	6/6	30·8 ± 12·0	6/6
M2	10·3 ± 0·3	0/2	288·5 ± 48·3	5/5	78·7 ± 50·2	3/4	69·2 ± 17·6	4/4	25·5 ± 13·8	3/3
C	9·1 ± 2·7	0/6	237·6 ± 58·9	6/6	32·7 ± 12·9	2/6	89·5 ± 23·2	6/6	7·8 ± 2·6	1/6
A	15·3 ± 3·6	0/6	211·2 ± 17·2	6/6	90·8 ± 7·7	0/6	51·6 ± 8·4	6/6	34·4 ± 12·5	6/6
W5	15·4 ± 4·5	0/6	200·3 ± 43·9	6/6	42·2 ± 9·4	5/6	93·4 ± 18·3	6/6	15·3 ± 6·2	6/6
W6	9·0 ± 2·2	0/6	181·1 ± 20·5	6/6	43·2 ± 16·3	0/6	54·3 ± 22·5	6/6	19·8 ± 16·3	6/6
W7	ND	ND	244·1 ± 33·9	6/6	46·3 ± 15·7	1/6	40·1 ± 10·4	6/6	9·7 ± 0·9	5/5

ND, no data.

[a]Samples with ELISA readings more than three times the mean value of the healthy control were considered positive and used to calculate the mean values in the table. The mean negative control values for each plate were subtracted from each experimental value and from the mean positive control value. These adjusted experimental values were expressed as a percentage of the adjusted positive control ± standard deviation.

[b]Number of samples DTBIA-positive relative to the number of samples TAS-ELISA-positive.

[c]The varieties tested were: Fino lemon (L), Star Ruby grapefruit (G), Clemenpons, Monreale and Commune clementines (P, M and C, respectively), Washington navel orange (W), sour orange (SO), Biondo Commune and Tarocco sweet oranges (B and T, respectively) and Avana mandarin (A).

CSPD substrate (although CSPD was preferred, to avoid the faint background sometimes observed in healthy controls when using the chromogenic substrate). CPsV was readily detected by DAS ELISA and TAS ELISA with either MAb in the four types of tissue assayed (data not shown).

To assess if CPsV could be reliably detected by DTBIA in different commercial citrus cultivars, plants of three sweet orange cultivars (Salustiana, Valencia Late and Washington navel), Okitsu satsuma, Nules clementine, Fortune mandarin, Ortanique tangor, Marsh grapefruit and Fino lemon were graft-inoculated with psorosis isolates P121, P126 and PB108, using two plants per isolate and variety. After symptom onset, young shoots from each inoculated plant and from a noninoculated control were sectioned, printed onto nitrocellulose membranes and analysed by DTBIA using a 1:1 (v:v) mix of MAbs 13C5 and 2A3. Previous experiments had shown that the isolate P121 is not recognized by MAb 2A3 (Alioto *et al.*, 1999). Tissue prints from all inoculated plants positively reacted with the antibody mix, regardless of the citrus variety and CPsV isolate combination, whereas no reaction was observed in tissue prints from uninoculated controls. ELISA readings obtained for each isolate in different hosts were similar (data not shown) and minor differences sometimes observed in DTBIA intensity were not consistent, suggesting that CPsV accumulates to equivalent levels in all of them.

Serological detection of CPsV in field-grown samples

To compare reliability of ELISA and DTBIA for CPsV detection in field-grown samples and determine possible seasonal limitations, 10 infected trees from Valencia, Spain (four Washington navel orange, two Clemenpons clementine, two Star Ruby grapefruit and two Fino lemon), and 10 from Naples, Italy (one sour orange, two Navelina and one Washington navel orange, one Tarocco and one Biondo Commune sweet orange, two Monreale and one Commune clementine, and one Avana mandarin), were selected and sampled in winter, spring and summer. Trees from Spain showed decline, poor performance and in some cases (sweet orange and grapefruit) bark scaling, whereas the Italian trees were not scaled (they were less than 10 years old) and generally looked normal, although sometimes young leaves showed chlorotic flecking in the spring. On each occasion, six old leaves and, when available, six young shoots were collected from each tree and analysed by DAS ELISA and TAS ELISA. Samples reacting

positively in DAS ELISA were also positive in TAS ELISA with either antibody and vice versa. These samples were then tested by DTBIA. Results obtained with each MAb were similar on both types of membrane, although 13C5 usually gave a weaker signal than the other antibody. Data obtained with 13C5 are summarized in Table 1.

The reaction patterns of the samples from the 10 Spanish and the 10 Italian trees were similar. Relative to the positive control, ELISA values from old leaves were generally below 50%, whereas young shoots from the same trees usually gave values two to eight times higher. Thirteen individual old leaves from the Spanish trees and 12 from the Italian trees (7·2 and 6·9%, respectively) failed to react in ELISA, whereas the corresponding numbers of false negatives using young shoots were five (4·2%) and three (2·5%), respectively (Table 1).

Detection by DTBIA varied with the season and plant part used for printing. Almost all prints from ELISA-positive young shoots taken in the spring were also positive by DTBIA. This coincidence between ELISA and DTBIA results was also observed in young summer shoots from the Italian trees and from many Spanish trees, but in the latter group, a few trees yielded variable results, and none of the shoots from the Spanish clementine trees (P1 and P2) were DTBIA-positive (Table 1). As indicated above, the Spanish trees were suffering from decline and produced few new shoots that hardened very soon. DTBIA reactions from old leaves were more variable and sometimes none of the prints prepared from a tree reacted positively (Table 1).

The proportion of old leaves reacting in DTBIA did not depend on the citrus variety, since variable reactions were observed in all of them, nor was there any correlation with the virus titre as detected by ELISA. For example, within the Spanish group of trees (Table 1), old leaves from tree W1 (Washington navel orange) gave mean relative TAS-ELISA values of 67·8% in winter and 70·2% in summer, but only one leaf in winter reacted positively in DTBIA. Conversely, old leaves from tree W4 (also Washington navel orange) gave, in the same seasons, relative TAS-ELISA percentages of 35·0 and 31·9%, respectively, but four leaves in winter and two in summer were positive in DTBIA. Similarly, within the Italian group of trees, old leaves from tree W5 (Washington navel orange) gave relative TAS-ELISA percentages of 15·4% in winter and 15·3% in summer, but, whereas all six leaves analysed in summer were DTBIA-positive, none taken in winter yielded a detectable reaction.

Comparison of serological and biological indexing in field trees

To assess the reliability of ELISA and DTBIA for diagnosing psorosis in comparison with symptom inspection in the field and biological indexing in the glasshouse, 47 field trees (22 Washington navel orange, 13 Nules clementine, three Sanguina, three Valencia and two Blanca sweet orange, one Willowleaf mandarin, one Owari satsuma, one Fino lemon and one sour orange) from the main citrus areas in eastern Spain were sampled in spring and analysed. Forty-one of them showed typical psorosis bark scaling in the trunk or limbs; one showed atypical bark scaling with a crater-like appearance in the trunk; one had severe concave gum symptoms (Guerri, 2000) with slight bark cracking at the edge of some concavities; three had no trunk symptoms but showed chlorotic flecking or spotting in young leaves; one was symptomless.

Table 2 Biological indexing of psorosis and serological detection of *Citrus psorosis virus* (CPsV) in samples from 47 field trees in eastern Spain

	Reaction patterns					
Diagnostic procedures	1	2	3	4	5	6
Field symptoms						
Bark scaling	+	+	−	+	−	−
Biological indexing						
Shock or leaf symptoms	+	+	+	+[a]	+[a]	−
Cross-protection	+	+	+	−	−	−
Serological indexing						
TAS ELISA 13C5	+	+	+	−	−	−
DTBIA 13C5	+	+	+	−	−	−
TAS ELISA 2A3	+	−	+	−	−	−
DTBIA 2A3	+	−	+	−	−	−
No. of trees showing each reaction pattern	39	2	2	1	1	2

[a]Leaf symptoms only.

From each tree, several young shoots were used to prepare tissue prints and were then pooled, trimmed and desiccated over silica gel for further analysis by DAS and TAS ELISA. Budsticks were also taken from each tree and used to graft-inoculate two Pineapple sweet orange and two *C. excelsa* seedlings for biological indexing. After symptom observation in two flushes, the sweet orange plants were challenge-inoculated with PB108 to test for cross-protection against psorosis B.

Results are summarized in Table 2. All samples reacting with A322 in DAS ELISA also reacted with MAb 13C5 in TAS ELISA (not shown), but two samples reacting with 13C5 were not recognized by MAb 2A3. For each MAb, CPsV detection by TAS ELISA and by DTBIA were always coincident. Duplicate membranes prepared at sampling time, stored at room temperature (20–25°C) in the dark and processed 1 year later, yielded the same results as those processed immediately.

Comparison of symptom expression at sampling time and biological and serological indexing showed six different reaction patterns. The 41 trees showing typical psorosis bark scaling (patterns 1 and 2), also induced shock and/or young-leaf symptoms in indicator plants, protected them against challenge with psorosis B and were ELISA- and DTBIA-positive. Thirty-nine of these trees reacted with both MAbs, whereas two failed to react with MAb 2A3. Two nonscaled trees with young-leaf symptoms (pattern 3) induced shock and young-leaf symptoms on indicator plants, protected them against psorosis B and reacted with antibodies as trees grouped in pattern 1. The tree showing atypical bark scaling (pattern 4) and the tree

showing concave gum symptoms (pattern 5) both induced chlorotic flecks and spots on young leaves of indicator plants, but they did not protect against psorosis B nor did they react with any of the antibodies. Finally, one nonscaled tree showing young leaf symptoms and the only symptomless tree sampled did not induce any reaction or cross-protection on indicator plants and neither tree reacted with CPsV-specific antibodies (pattern 6).

Discussion

The results show that CPsV can be readily and specifically detected by ELISA in any season and by DTBIA when young tissue is available. Both assays allowed detection of many CPsV sources from Spain and Italy in different citrus species and varieties, grown under glasshouse conditions or in the field. The biological characteristics of these sources were variable and their symptoms ranged from only mild flecking or spotting on young leaves to psorosis B pustules and blisters.

However, while ELISA allowed reliable CPsV detection even in suboptimal conditions, selection of the correct tissue was critical for DTBIA detection. CPsV was consistently detected in tissue prints of young tender leaves or shoots, but erratically in those prepared from old hardened leaves. This would limit the use of DTBIA when suitable tissue is not available, particularly in winter.

Although ELISA data from field trees indicate that the amount of virus is generally lower in older leaves than in young shoots, antigen titre may not be the only factor limiting DTBIA detection. For example, in field trees, the number of old leaves reacting in DTBIA was not correlated with ELISA values, and in glasshouse plants, old leaves usually gave a weak DTBIA reaction even if their ELISA values were often similar to those of young leaves (data not shown). Most likely, it is the juiciness of the tissue and thus the amount of antigen blotted on the membrane that limits CPsV detection by DTBIA. This would explain the inconsistent detection observed in tissue prints from old leaves or summer shoots from psorosis-declined trees, which become quickly hardened. It might also be the reason for easier detection of CPsV in young ovaries than in other flower parts or in leaves, as recently reported by D'Onghia *et al.* (2001).

The results confirm the previous finding that MAb 2A3 does not react with certain isolates, whereas 13C5 seems to recognize a more universal epitope (Alioto *et al.*, 1999). MAb 13C5 has so far reacted with all psorosis sources tested, including 14 isolates maintained in the IVIA collection (unpublished data) and numerous field samples from various citrus areas. However, to reduce the risk of missing potential isolates not recognized by either antibody singly, a 1:1 (v:v) mix of both antibodies was also used. This test detected several isolates not recognized by 2A3 but has not as yet detected any new isolate not recognized by 13C5 (data not shown).

Although CPsV is now widely assumed to be the cause of psorosis, the aetiology of this disease has not yet been demonstrated, and various agents or factors might contribute to it. A major deficiency of previous papers on the serological detection of CPsV (García *et al.*, 1997; D'Onghia *et al.*, 1998; Potere *et al.*, 1999; Alioto *et al.*, 1999, 2000; D'Onghia *et al.*, 2001) is that virus detection was not properly correlated with the presence or absence of psorosis disease as defined by field symptoms, biological indexing and cross-protection (Roistacher, 1993). In this work, CPsV detection by ELISA and DTBIA perfectly coincided with diagnosis based on biological indexing, particularly with the ability to cross-protect against psorosis B. Two trees that had not yet developed bark scaling were shown to be psorosis-infected by biological indexing and to contain CPsV by ELISA and DTBIA. Conversely, two nonscaled trees showing concave gum and young-leaf symptoms, respectively, did not contain CPsV and were shown to be psorosis-negative by biological indexing. Finally, one tree with atypical psorosis-like bark scaling was diagnosed as psorosis- and CPsV-free by biological and serological indexing, respectively.

These findings show for the first time that serological tests reliably reflect psorosis infection. The results also show that diagnosis based on field symptoms, including bark scaling, or on symptoms induced in young leaves of indicator plants, can be misleading. Cross-protection against psorosis B, considered the most reliable test for psorosis (Roistacher, 1991, 1993), is expensive and lengthy (taking about 8 months) and requires a temperature-controlled glasshouse and specialized personnel. The data in this paper show that cross-protection yields the same results as ELISA or DTBIA using tissue prints from young tender shoots.

Serological tests of field trees showed that in the worst conditions (old hardened leaves in winter), CPsV in infected trees was still clearly detected in at least one-third of the leaves sampled. This indicates that, in any season, high reliability can be achieved by using a minimum of four leaves taken from around the tree. However, DTBIA has the advantage of being simpler, cheaper and faster than ELISA (Cambra *et al.*, 2000) and can give the same specificity and sensitivity if tissue is selected appropriately (young tissue in early spring through to mid-autumn in the Mediterranean area). Tissue prints can be prepared in the field and stored for long periods without loss of reactivity. This possibility is very convenient for epidemiological studies in which large areas away from the laboratory are to be sampled in a short period. It also allows several membranes to be tissue-printed with the same shoots for future processing with new antibodies or membranes to be mailed to a different laboratory where these antibodies are available.

Although serological tools for CPsV diagnostics are now available, biological indexing of mother plants on sweet orange seedlings will continue to be used in certification programmes, as this is still the only procedure to detect other citrus pathogens, such as concave gum, impietratura or cristacortis (Navarro, 1993).

Acknowledgements

We thank M. E. Martínez and M. Boil for technical assistance in the laboratory and Jaime Piquer for excellent care

of plants in the glasshouse. SM received a fellowship from the Consellería de Cultura, Educación y Ciencia de la Generalitat Valenciana. This work was supported in part by the INCO-DC Program of the European Commission DG-XII (contract no. ERBIC18CT960044) and by INIA project SC97103.

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