Journal of Plant Pathology (2006), **88** (2), 171-177 *Edizioni ETS Pisa, 2006* 171

SEROLOGICAL DETECTION OF *CITRUS PSOROSIS VIRUS* USING A POLYCLONAL ANTISERUM TO RECOMBINANT VIRUS COAT PROTEIN

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SUMMARY

Citrus psorosis virus (CPsV) is the putative agent of psorosis, a widespread and economically important disease of citrus. This virus is erratically transmitted to herbaceous hosts in which it multiplies to low titer. It can be purified only with difficulty, which makes the production of antisera laborious. To overcome this impairment, the coat protein gene of an Italian virus isolate was cloned and sequenced. This sequence, which showed 97% identity at the amino acid level with a Florida isolate of CPsV, was expressed in *Escherichia coli* BL21 as a fusion protein with maltose binding protein. This recombinant protein was used as an antigen for immunization. The resulting antiserum successfully and specifically recognized CPsV in Western blots, immunoelectron microscopy and different ELISA protocols, using diverse tissues from citrus accessions from different geographical areas.

Key words: Citrus, *Citrus psorosis virus*, diagnosis, ELISA, ISEM, recombinant proteins, sequencing.

INTRODUCTION

Citrus psorosis virus (CPsV), the type species of the genus *Ophiovirus* (Vaira *et al.*, 2005), is the putative causal agent of psorosis, a major graft-transmissible disease of citrus (Roistacher, 1993). The CPsV genome consists of at least three single-stranded RNAs of negative polarity that have been totally sequenced (Barthe *et al.*, 1998; Sanchez de la Torre *et al.*, 2002; Naum-Onganía *et al.*, 2003; Martín *et al.*, 2004). RNA-3 encodes the *ca.* 49 kDa coat protein (CP) (Sanchez de la Torre *et al.*, 1998).

Routine detection of CPsV by laboratory methods, serology in particular, is a primary requirement for large-scale surveys such as those carried out for sanitary selection in the framework of certification programmes. Both monoclonal antibodies (Mab) and polyclonal antibodies (Pab) have been used to detect CPsV by ELISA or by direct tissue blot immunoassay (DTBIA) (García *et al.*, 1997; Alioto *et al.*, 1999; Potere *et al.*, 1999; D'Onghia *et al.*, 2001). Their production, however, has been impaired by the difficulty of obtaining sufficient clean antigen for immunization, due to the erratic transmission and low concentration of the virus in herbaceous hosts (Roistacher, 1993).

In this paper, we report the successful use of the recombinant protein strategy to raise a polyclonal antiserum directed to a recombinant viral coat protein (CP), and that the antiserum proved suitable for routine use in ELISA.

MATERIALS AND METHODS

Plant material, RT-PCR and cloning. Total RNAs were extracted from 100 mg of infected citrus leaves using microchromatography on silica particles, as described by Foissac *et al.* (2000). Tissue samples were from a citrus accession, infected by a CPsV isolate denoted ps110 (Table 1), maintained at collection of the University of Bari. DNA complementary to the viral CP gene was obtained by reverse transcription. Two overlapping fragments of the CP cistron were amplified from the CPsV isolate ps110, using primers CPV5'h (5' AAGAAGGATCCATGTCGATTCCTATTAAAGTGTC 3', nt –11 to 22, containing a *Bam* HI site), CPV1int (5' CCATCATACAGTTTTGACTTGACAAATTCAGG 3', complementary to nt 688 to 719), CPV1 (5' GCTTCCTGGAAAAGCTGATG 3', nt 654 to 673) and CPV3'c (5' CAGTAAGCTTTTAAAAGCATAACATGCAAGCACA 3', complementary to nt 1342 to 1366, containing a *Hind* III site), designed on the nucleotide sequence of the CPsV-4 strain from Florida (Barthe *et al.*, 1998; accession number AF036338).

For cDNA synthesis, 2.5 µl of total RNA extract (500 ng) and 0.5 µl of random primers (0.5 µg/µl) were denatured for 5 min at 95°C and chilled on ice. AMV-RT buffer (50 µl) (Promega, Madison, USA), 1.25 µl of 2.5 mM dNTPs, 1 µl RNasin (40 U/µl) and 0.4 µl AMV-RT (10U/µl) were added and the samples were incubated

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Table 1. CPsV isolates in citrus accessions from the collection of the University of Bari. In bold the ps110 isolate used for sequencing.

Virus isolate	Geographical origin
ps101, ps104, ps105, ps106, **ps110,** ps112, ps114, ps115, ps116, ps117, ps118, ps120, ps124, ps126, ps127, ps130, ps131, ps134, ps137, ps140, ps141, ps142, ps144, ps145 194-X, 195-X (a), 195-X (b), 204-X, 208-X, 310-X, 311-X, 320-X, 325-X, 365-X, 391-X (a), 391-X(b), 391-X(c), 392-X, 394-X, 3V, 10V, 36V, 72V, 77V(a), 77V(b), 86V, lot4F2P90, lot3F1P450, lot57N1148, lot 97N1148 Total = 51	Italy
317-X, 318-X(a), 318-X(b) Total = 3	Spain
243-X(a), 243-X(b), 244-X(a), 244-X(b), 245-X(a), 245-X(b), 246-X(a), 246-X(b), 247-X(a), 247-X(b), 248-X(a), 248-X(b), 249-X, 252-X, 254-X Total = 15	USA
205-X, 312-X, 314-X(a), 314-X(b), 388-X Total = 5	Unknown origin

for 1 h at 42°C. Five µl of cDNA were added to 25 µl of a PCR cocktail containing 1 x PCR buffer (Roche Applied Science, Basel, Switzerland), 1.25 µl of dNTPs 2.5 mM, 1.25 µl of each forward and reverse primer (6 µM stock) and 1 U of *Taq* polymerase. PCR conditions were: 1 cycle of 5 min at 94°C; 35 cycles of 30 s at 94°C, 40 s at 53°C and 40 s at 72°C; 1 cycle of 7 min at 72°C.

Primers CPV5'h/CPV1int and CPV1/CPV3'c were used to amplify the 5'-terminal region (730 nt) containing the ATG codon and the 3'-terminal region (722 nt) of the CP gene, respectively. Amplification products, detected by 1% agarose electrophoresis and ethidium bromide staining, were purified using QiaquicK Gel Extraction Kit (Qiagen, Valencia, USA), ligated to pGEM-T easy plasmid (Promega, Madison, USA) and transformed into *Escherichia coli* JM110.

Nucleotide sequences of the CP half clones were assembled with DNA STRIDER program (Marck, 1988) and compared with the existing sequence of the isolate CPsV-4 using FASTA (Altschul *et al.*, 1997).

To obtain the complete CP sequence, the two halves of the CP gene were excised by *Eco* RI digestion and the resulting fragments were ligated at a unique *Eco* RII site in the overlapping 66 nt region. The complete CP gene was amplified with primers CPV5'h/CPV3'c and cloned in pGEM-T-easy vector.

Protein expression and production of polyclonal antiserum As-Ps.Rc1. The CP gene was ligated into the *Bam* H1 and *Hind* III sites of the protein expression vector pMAL-c2X (New England Biolabs, Beverly, USA) and products were used to transform *E. coli* BL21 and DH5α cells. Recombinant plasmids were further analysed for sequence confirmation.

The maltose-binding protein (MBP)-CPsVCP recombinant protein was expressed and purified using affinity chromatography, according to the manufacturer's manual (New England Biolabs, Ipswich, USA) and eluted in 1 ml fractions with column buffer containing 25 mM NaCl and 10 mM maltose. Each fraction was checked for the presence of recombinant protein by SDS-PAGE in 10% gels (Laemmli, 1970).

A New Zealand rabbit was immunized with 1 mg of the recombinant protein in Freund's incomplete adjuvant as the first intramuscular injection, followed at weekly intervals by two subcutaneous injections, each with 0.5 mg antigen. Six bleedings were made every week after the last injection. Antibodies, denoted As-Ps.Rc1, were purified from the resulting antiserum by Fast Perfomance Liquid Chromatography (FPLC) (Amersham.Biotech, Uppsala, Sweden). The titre of As-Ps.Rc1 was determined by gel double diffusion tests (GDT) (0.7% agar, 0.85% NaCl and 0.02% sodium azide).

Western blotting. Samples (100 mg) from infected leaves of citrus or *Chenopodium quinoa* were ground in 2.5 volumes of extraction buffer (0.5 M Tris-HCl pH 8.8, 2% SDS, 40% sucrose, 4% 2-mercaptoethanol), the extracts were boiled for 3 min and centrifuged at 4000 rpm for 4 min. Aliquots of 5 µl of the supernatant fraction were analysed by electrophoresis in 10% SDS slab gels together with recombinant CPsV CP (5 µg/µl l) and 1 µg CP from a Laemmli buffer-denatured virus preparation purified from *C. quinoa*.

Gels were then electroblotted on polyvinyl difluoride membranes (PVDF, Immobilon-P, Millipore, Billerica, USA). Following blocking with 1% BSA, 5% not-fat dry milk, 0.05% Tween-20 in TBS buffer 1X for 2 h at room temperature, membranes were incubated for 1h with crude As-Ps.Rc1 serum diluted 1:2000, 1:1000 or 1:500. After 3 washes of 10 min each in 1X TBS, 0.1% Tween-20, the membranes were incubated for 30 min with a 1:12000 dilution in blocking solution of a goat antirabbit IgG-AP conjugate (Sigma, St. Louis, USA). Three sequential washing steps of 15 min each were followed by chemiluminescent detection with 1 ml of CDP-star substrate (1:100) (Roche Applied Science, Basel, Switzerland). Protein bands were detected by exposure to X-ray films for 30 min.

Immunosorbent electron microscopy (ISEM). Extracts for ISEM (Milne, 1993) were prepared by crushing leaf tissues from healthy and infected *C. quinoa* in 0.1 M phosphate buffer, pH 7.2. Carbon-formvar coated grids were floated for 15 min at room temperature, on a drop of crude antiserum diluted 1:500 in phosphate buffer, rinsed with 0.05 M phosphate buffer, pH 7.2, drained and then floated on the plant extract at 4°C overnight. After three washes in phosphate buffer, grids were floated at room temperature for 15 min, on a drop of 1:40 and 1:80 dilutions of the same antiserum, rinsed with distilled water, stained with 2% uranyl acetate and examined in a Philips Morgagni electron microscope.

ELISA. IgGs purified by FPLC were used for DAS- and DASI-ELISA (Clark and Adams, 1977) with citrus leaf tissues (100 mg) homogenized in 1 ml of extraction buffer (20g/l PVP-24000, 0.5 ml/l Tween-20, 2% nonfat dry milk, in PBS 1X, pH 7.4).

For DAS-ELISA, plates were coated for 2 h at 37°C with a 2 µg/ml or 1.5 µg/ml of purified IgGs, before the addition of alkaline-phosphatase-conjugated As-Ps.Rc1 IgGs, diluted 1:250. For DASI-ELISA, plates were coated for 2 h at 37°C with a 1:12,500 dilution of a mix of 19 monoclonal antibodies (Potere *et al*., 1999) and, after addition of antigens, purified CPsV CP IgGs were used as secondary antibodies at concentration of 1 µg/ml. Alkaline-phosphatase-conjugated goat antirabbit IgGs, diluted 1:10000 were added and plates were incubated at 37°C for 2 h.

In all cases, extracts were incubated in ELISA plates at 4°C overnight prior to the addition of substrate (1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8). As reference control, a DAS-ELISA test was made with a cocktail of 19 monoclonal antibodies and a conjugated Mab Ps-29 (Potere *et al*., 1999), which is used routinely in our laboratory to detect CPsV. A reaction was considered positive when the mean absorbance value for any given sample read with a microplate ELISA reader was greater than twice the mean value of healthy controls.

Dot tissue blot immunoassay (DTBIA). This assay was done essentially as described by D'Onghia *et al.* (2001). Transversely cut tender shoots, or whole flowers, or rolled old and young leaf blades were gently pressed onto nitrocellulose membranes with a 0.45-µm pore size (Bio-Rad, Hercules, USA). Prints were allowed to dry for 30 min at room temperature, placed in a 1% BSA and incubated for 2 h at room temperature in a plastic container on a shaker stirrer. After washing with PBS-Tween 20, blotted membranes were incubated for 2 h with alkaline-phosphatase-conjugated As-Ps.Rc1 IgGs at a 1:500 dilution. Membranes were incubated in substrate made by dissolving a tablet of BCIP-NBT Sigma fast in 10 ml distilled water until a purple-violet colour appeared in the positive control.

RESULTS

Cloning and protein expression. RT-PCR of total RNA extracts from citrus leaves infected by CPsV isolate ps110 (Table 1) yielded two amplicons of 730 and 722 bp respectively that overlapped by 66 bp, which were cloned separately in pGEM-T-easy, and sequenced. The resulting complete CP sequence (Genbank, accession number AM159538) showed a 85% and a 97% identity with that of the Florida isolate CPV-4, at the nucleotide and amino acid levels, respectively. The two CP inserts were assembled in pMal-c2X to obtain plasmid ps110cpMBP, which was used to transform *E. coli* DH5α and BL21, for protein expression.

The best conditions for expression were obtained with strain BL21 using an isopropyl-b-D-thiogalactoside (IPTG) concentration of 0.3 mM and 3 h of induction at 37°C (Fig. 1). In DH5α cells, the expressed protein was insoluble and associated with sonicated bacterial pellets (not shown). Western blot analysis showed that truncated forms of the CPsV-MBP protein were present in the induced bacteria, together with the full length fusion protein with the expected size of 92 kDa (43 kDa MBP plus 49 kDa viral CP) (Fig. 2). The yield of recombinant protein was *ca.* 30 mg/l of culture.

Production of the polyclonal antiserum As-Ps.Rc1 and Western blotting. The antiserum raised had a titre of 1:128 as determined by GDT and did not react visibly with healthy plant extracts. All six bleedings were assayed for their suitability for use in Western blot (WB) and ELISA. Best results in both assays were obtained with the antiserum from the 4th bleed. Antisera

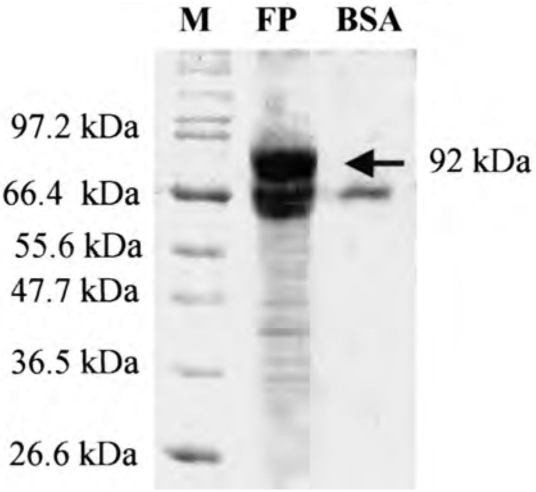

Fig. 1. 10% SDS-PAGE of fusion protein (FP) after elution, stained with Coomassie brillant blue. BSA: bovin serum albumin. M: broad range molecular weight protein standards.

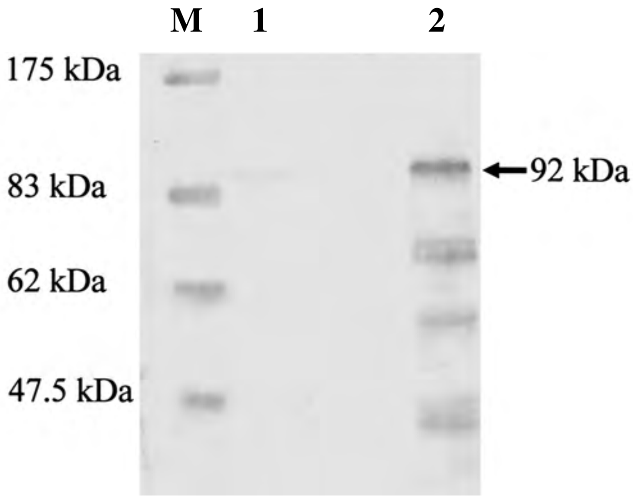

Fig. 2. Western blot analysis with serum antiMBP (1:12000) against total protein extracts from clone ps110cpMBPBL21 BL21 before (1) and after induction (2) at 37°C. M: prestained range molecular weight protein standards.

from the 1st, 2nd and 3rd bleeds did not give a visible reaction in Western blot, while in ELISA showed an unsatisfactory sensitivity (not shown). Antisera from the 5th and 6th bleeds did not increase the sensitivity and gave a high background.

Western blots using the antiserum from the 4th bleed diluted 1:1000 detected a protein with the expected size for CPsV coat protein (49 kDa) in total protein extracts from infected *C. quinoa* tissues and in virus preparations purified from leaves of *C. quinoa* infected with the ps110 isolate (Fig. 3). The antiserum also detected MBP-CP fusion protein and MBP alone, but did not react with total proteins extracted from healthy *C. quinoa* leaves (Fig. 3), thus confirming its specificity for CPsV CP.

ISEM. The antiserum, diluted 1:40 and 1:80 in phosphate buffer, clearly decorated virus particles trapped from crude extracts of ps110-infected *C. quinoa* leaves (Fig. 4).

ELISA. Preliminary tests, using As-Ps.Rc1 IgGs, were done in comparison with a routine DAS-ELISA format with Mab Ps-29 (Potere *et al*., 1999). The results (Table 2) showed that As-Ps.Rc1 successfully detected CPsV in citrus leaves in both ELISA formats tested: (i)

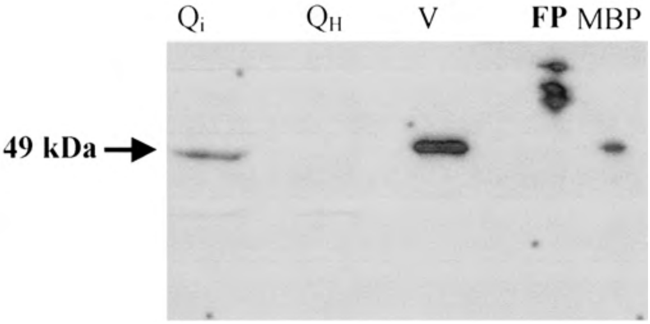

Fig. 3. Western blot analysis with IV° As-Ps.Rc1 serum bleed (1/1000) against fusion protein (FP), purified virus from *C. quinoa* infected with ps110 isolate (V), total protein extracted from healthy *C. quinoa* (Qh), total protein extracted from infected *C. quinoa* with ps110 (Qi), maltose binding protein (MBP). Chemiluminescent detection.

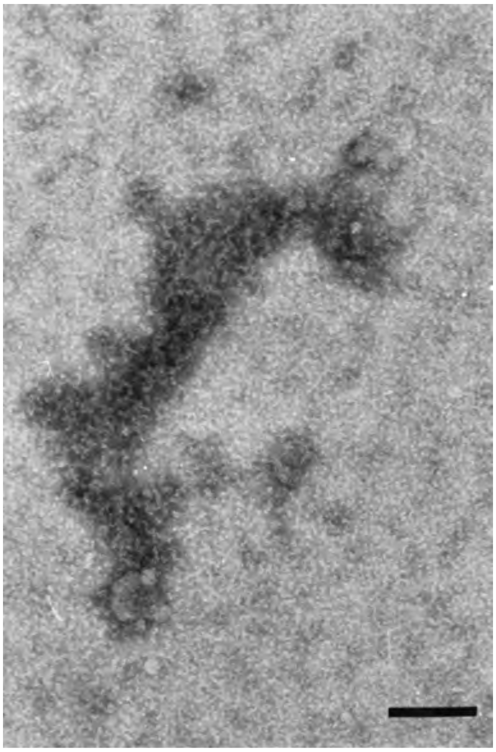

Fig. 4. Stained particles of virus isolate ps110 after exposure to As-Ps.Rc1 serum for decoration. Bars = 100 nm.

DASI-ELISA in which plates were coated with monoclonal antibodies diluted 1:12500, As-Ps.Rc1 IgGs (1µg/ml) were used as secondary antibody followed by alkaline phosphatase conjugated antirabbit IgGs at 1:10000 dilution, (ii) DAS-ELISA using purified IgGs of As-Ps.Rc1 (1.5-2 µg/ml) for plate coating and alkaline phosphatase-conjugated purified IgGs As-Ps.Rc1 (1:250). DASI-ELISA appeared to be more sensitive than DAS-ELISA (As-Ps.Rc1). This difference in sensitivity was evident in assays of a different CPsV isolate (ps101), which was clearly positive in DASI-ELISA (I/H= 6.7), but close to the threshold value in DAS-ELISA (I/H= 2.3). Therefore DASI-ELISA was compared with the routine DAS-ELISA (Mab Ps-29) to test the complete citrus collection listed in Table 1, including accessions infected by CPsV isolates coming from different geographic areas (Djelouah *et al.,* 2000; Loconsole *et al.*, 2002).

All 74 isolates were detected at least once by both DAS Mab Ps-29 and DASI As-Ps.Rc1 during surveys carried out monthly from April to July 2004. The failure to detect some of them was probably due to the uneven distribution of the virus in the plants. Tests in April gave the best results since both ELISA systems detected 54 (72.9%) isolates. Among the remaining isolates, seven were detected by DAS-ELISA Mab Ps-29, whereas a different seven isolates were detected by DASI-ELISA As-Ps.Rc1 (82.4% in both cases). These findings suggest that in this period of the year both reagent formats were equally effective in virus detection.

Detection of CPsV in field samples. To check on the reliability of CPsV detection by ELISA throughout the year, old leaves, young leaves, and flowers (when available), were collected monthly from infected plants of two sour oranges, one clementine, one Satsuma Myiagawa and one Naveline, grown in an Apulian (southern Italy) grove. CPsV infection of these sources had been ascertained by DAS-ELISA with Mabs and dot-hybridization (Loconsole *et al.,* 2002). Table 3 shows that serological detection of CPsV was possible virtually throughout the year from one organ or the other, and that there was no significant difference in the performance of the two ELISA systems. Highest detection rates were obtained in spring, from flowers and young leaves, and in autumn from the young flush vegetation.

DTBIA. CPsV was readily detected by tissue printing from flowers, tender shoots, and young leaves collected in spring but not from hardened leaves (not shown).

DISCUSSION

As reviewed by Alioto *et al.* (2006), a number of serological reagents for the detection of CPsV have been developed in the last 15 years or so. Polyclonal antisera and monoclonal antibodies are currently available, but their production is made laborious by the difficulty of obtaining enough antigen in a purified form for immunization. CPsV is indeed difficult to transmit and maintain on herbaceous hosts, on which it multiplies to low concentrations. Moreover many CPsV isolates are not mechanically transmissible from infected orange to other citrus or herbaceous hosts (Roistacher, 1991; 1993).

Recombinant DNA technology may help to circumvent these problems. In recent years, several authors have reported the successful use of antibodies produced to recombinant coat proteins (Ling *et al.*, 2000; Abou-Jawdah *et al.*, 2004). In this work, the CP gene of an Italian isolate of CPsV, that was almost identical in CP amino acid sequence to a Florida CPsV isolate, was cloned and expressed as fusion protein with MBP in bacterial cells. Polyclonal antiserum to the fusion pro-

Table 2. Results of ELISA tests in which the efficiency of the polyclonal antiserum As-Ps.Rc1 (DASI, DAS) and the monoclonal antibody Mab Ps-29 (DAS) was compared for the detection of CPsV isolates ps110 and ps101 in crude citrus extracts.

Samples	DASI (As-Ps.Rc1)		DAS (As-Ps.Rc1)		DAS (Mab Ps-29)[a]	
	A405[b]	I/H[c]	A405[d]	I/H	A405[e]	I/H
ps 110	1.6	26.7	0.9	15.0	2.0	33.3
ps101	0.4	6.7	0.14	2.3	0.235	3.92
Healthy	0.06	1	0.06	1	0.06	1

[a] Routine DAS-ELISA with Mab Ps-29, as reference control.

[b] Readings of A405 were measured after 30 min of substrate incubation.

[c] Ratio of absorbance values of infected samples/healthy control (I/H). Values below 2 indicate negative reactions.

[d, e] Readings of A405 were measured after 2h of substrate incubation.

Table 3. Results of monthly tests with two ELISA protocol on aged leaves (A), young leaves (B), and flowers (C) collected from field-grown citrus plants.

	Feb 2004	Mar 2004	Apr 2004	May 2004	Jun 2004	Jul 2004	Aug 2004	Sep 2004	Oct 2004	Nov 2004	Dec 2004	Jan 2005
Aged leaves												
DAS (Mab Ps-29)	1/5*	4/5	0/5	3/5	3/4	nt	nt	0/5	1/5	nt	2/5	3/5
DASI (As-Ps.Rc1)	2/5	4/5	1/5	3/5	3/4	nt	nt	0/5	2/5	nt	2/5	1/5
Young leaves												
DAS (Mab Ps-29)	nt	2/3	5/5	5/5	4/4	nt	nt	4/4	1/2	nt	nt	nt
DASI (As-Ps.Rc1)	nt	2/3	5/5	5/5	4/4	nt	nt	3/4	2/2	nt	nt	nt
Flowers												
DAS (Mab Ps-29)			4/5	5/5								
DASI (As-Ps.Rc1)			4/5	5/5								

* Infected samples/tested samples; nt = not tested.

tein (As-Ps.Rc1) and IgGs purified from it were used successfully in Western blotting, ISEM, various ELISA protocols, and DTBIA for the detection of antigen in total protein extracts, crude sap from infected *C. quinoa* or citrus, purified virus, and tissue prints. All these tests showed that the As-Ps.Rc1 antiserum was specific and able to detect CPsV in infected plants without preabsorption with healthy plant antigens.

The suitability of As-Ps.Rc1 antibodies for use in DASI-ELISA was compared with a validated Mab ELISA kit (Potere *et al.*, 1999) and shown to be equally effective in recognizing the homologous isolate and a number of other isolates with both ELISA protocols.

When the comparison was extended to 74 virus isolates from different countries, the results (Table 2) indicated that detection rate by both ELISA systems was the same (73%) in April; a period in which virus concentration in flowers and young leaves is probably higher, as reported by Martín *et al.,* (2002). Furthermore, assays from field-grown citrus plants showed that As-Ps.Rc1 detected CPsV by DASI-ELISA in all organs (aged and young leaves, flowers) analysed throughout the year, similar to results of DAS-ELISA Mab Ps-29. The surveys indicated that young leaves and flowers collected in April, May and June (Table 3) were better tissue than hardened leaves for CPsV detection. Finally, As-Ps.Rc1 was reliably used for virus detection in young tissues by DTBIA.

These results support the notion that the availability of an antiserum against recombinant viral CP represents an additional and/or alternative tool for manufacturing standardized kits for the serological detection of CPsV. The DASI-ELISA system developed is a clear improvement since it allows the use of As-Ps.Rc1 IgGs in place of the conjugate monoclonal antibody Mab Ps-29. Furthermore the use of recombinant coat proteins eliminates the need to maintain and purify "difficult" viruses and makes it possible to standardize the process of antigen and antiserum production.

ACKNOWLEDGEMENTS

This work was supported by MiPAF Project "Ricerche e Sperimentazione nell'Agrumicoltura Italiana". Paper n. 158. Grateful thanks are expressed to Dr. P. Saldarelli for useful suggestions and comments and to Prof. Martelli for revising the manuscript.

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Received 29 November 2006
Accepted 26 April 2006