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Improved detection of citrus psorosis virus using polyclonal and monoclonal antibodies

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Citrus psorosis is a serious and widespread disease associated with *citrus psorosis virus* (CPsV), a novel filamentous negative-stranded virus in the genus *Ophiovirus*. Laborious and costly indexing on test plants has been the only routine diagnostic method available, but recently an antiserum usable in double antibody sandwich (DAS) ELISA has been prepared. Here, major improvements to the DAS-ELISA protocol, a new purification method, and production of two monoclonal antibodies (mabs) to CPsV, an IgG and an IgM are reported. A highly sensitive triple antibody sandwich (TAS) ELISA making use of the mabs is described. In glasshouse citrus the homologous virus was still detectable at a tissue dilution of 1/6250 in DAS and at 1/31250 in TAS-ELISA. Both the DAS and IgG mab-TAS formats detected all CPsV isolates so far tested (from Argentina, Italy, Lebanon, Spain and the USA). A few isolates were not detected by the IgM mab.

Keywords: *citrus psorosis virus*, DAS-ELISA, *Ophiovirus*, serology, TAS-ELISA

Introduction

Citrus psorosis disease is characterized by bark scaling in the trunk and branches of adult citrus trees, often leading to decline and reduced yield. It is readily graft-transmitted but symptoms rarely appear before trees are 10 years old, so growers often propagate psorosis-infected buds from symptomless trees. The disease has thus become widespread in the Mediterranean basin, South America and probably in Asia. Natural field spread does not appear to be a problem in the Mediterranean basin but there is evidence for natural spread in California, Texas and Argentina (Roistacher, 1993). The presumed causal agent is now partially characterized as a new virus, *Citrus psorosis virus* (CPsV), placed in a new genus, *Ophiovirus* (Milne *et al*., 1999).

Psorosis has not been controlled or even clearly identified except in a few places such as California, Spain and South Africa, where indexing on indicator plants is carefully applied. The problem is that indexing is slow and costly, and requires trained and dedicated personnel. Inexpensive, simpler and more rapid diagnostic methods are urgently required and would be of great value for understanding the extent and dynamics of the disease, and for routine indexing and replanting with psorosis-free material.

An early antiserum to the virus (da Graça *et al*., 1991) was not suitable for enzyme-linked immuno-sorbent assay (ELISA), although useful work was done with it by Western blotting (Navas-Castillo *et al*., 1993; Navas-Castillo & Moreno, 1995). The first available monoclonal antibodies (mabs) (Derrick *et al*., 1993) were of no value as a diagnostic reagent, as reported by Barthe *et al*. (1998).

An antiserum to the CRSV-4 isolate of the virus (Garcia *et al*., 1997) could be employed for DAS-ELISA, and was used to show that ELISA data correlated closely with the results of indexing on indicator plants, confirming the strong link between classical psorosis symptoms and CPsV, and indicating that at least in the Apulia region of southern Italy the virus is widespread (D'Onghia *et al*., 1998). However, this DAS-ELISA was not very sensitive and depended on careful absorption of the antiserum with healthy plant material. Recently, mabs to an Italian isolate of CPsV (Djelouah *et al*., 1999) have been prepared and used to differentiate among CPsV isolates from different parts of the world, but data are not available on the sensitivity of detection achieved.

Here it is shown that modifications to the DAS-ELISA protocol used by Garcia *et al*. (1997) and D'Onghia *et al*. (1998) can greatly improve detection limits. Also reported are the preparation of mabs for the CRSV-4 isolate and their use in TAS-ELISA. This system further increases

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detection sensitivity. One of the mabs reacted with all CPsV isolates tested.

During the course of this work it was found that the improved protocols developed for CPsV also gave better results with other viruses, and so may be applicable more widely. The protocols are therefore discussed in some detail.

Materials and methods

Virus isolates

The CRSV-4 isolate of CPsV (Garnsey & Timmer, 1980), provided by K. S. Derrick (Citrus Research and Education Centre, Lake Alfred, FL, USA), was used for antibody production. Other CPsV isolates used in this work are listed in Table 1. All these isolates except Na-1 (not indexed) have been biologically indexed as psorosis-positive (Garnsey & Timmer, 1980; da Graça *et al*., 1991; Navas-Castillo & Moreno, 1995; D'Onghia *et al*., 1998; Garcia *et al*., 1997; C. N. Roistacher, Department of Plant Pathology, University of California, personal communication 1996).

Plants and plant material

CRSV-4 and several other isolates were maintained in Madam Vinous sweet orange in pots in a glasshouse held at approximately 25/18°C (day/night). Other isolates of CPsV were received as leaf material dried over silica gel or calcium chloride.

For mechanical inoculation to *Chenopodium quinoa*, young citrus leaves infected with CRSV-4 and bearing symptoms were homogenized in ice-cold buffer (50 mM phosphate pH 7·0, 5 mM DIECA, 1 mM EDTA, 5 mM sodium thioglycolate) with addition of 0·25% (w/v) active carbon, and inoculated immediately. To obtain systemic infection in *Gomphrena globosa* plants, *C. quinoa* local lesion material was mechanically inoculated as above, whereas mechanical transmission between *G. globosa* plants was best obtained using TACM buffer (Derrick *et al*., 1988) plus active carbon. Highest virus concentrations (estimated by EM negative staining and by ELISA) were obtained in *G. globosa* plants held at about 30/25°C (day/night) with supplementary lighting to give an 18-h day.

To compare the results of DAS and TAS-ELISAs performed at different times, and to provide kits to other workers, standard extracts were prepared as follows. Healthy or infected leaves of *G. globosa* were homogenized in 3 volumes (w/v) of phosphate-buffered saline containing 0·05% Tween-20 (PBS-T) and 2% polyvinylpyrrolidone (PVP). The extract was filtered through nylon stocking and centrifuged at low speed. The supernatants were lyophilized. Tests showed that infectivity was lost, so the samples presented no quarantine hazard, but antigenicity was maintained over a period of months.

Table 1 ELISA results on 34 isolates of CPsV of different origin. Infected/healthy optical density ratios of 3 or more were taken as positive. Each test was repeated at least once on a different sample

		TAS	
Isolates	DAS	IgG	IgM
North American			
CRSV-4	+	+	+
P200	+	+	+
P201	+	+	+
P203m	+	+	+
P205	+	+	+
P208	+	+	+
P209	+	+	+
P213	+	+	+
P215m	+	+	+
P216m	+	+	+
Argentine			
CPsV 173–22	NT	+	+
CPsV 90–1-1	NT	+	+
CPsV 189–34	NT	+	+
CPsV 504–5	NT	+	+
CPsV 100–40–1	NT	+	+
CPsV 100–16–5	NT	+	+
Italian			
ITA-3	+	+	+
NA-1	+	+	+
IAM-197X	+	+	+
IAM-393X	+	+	+
IAM-7V	+	+	+
IAM-320X	+	+	–
IAM-398X	+	+	+
Lebanese			
IAM-165X	+	+	+
Spanish			
Sp2	+	+	+
RS-SOR	+	+	+
RS-SR	+	+	+
P-121	+	+	–
P-126	+	+	+
P-132	+	+	+
P-129	+	+	+
RS-105	+	+	–
RS-108	+	+	+
PB-102	+	+	+

NT, not tested.

Virus purification

For production of mabs, the virus was purified according to Garcia *et al*. (1997) with modifications. Briefly, infected *G. globosa* leaves were homogenized in 10 volumes (w/v) of TACM buffer plus active carbon. The solvent-clarified extract was given two cycles of precipitation with 10% polyethylene glycol (PEG), 1% NaCl and 0·1% Nonidet P40. The resulting preparation was centrifuged into a 10–40% caesium sulfate density gradient in TACM buffer at 310 000 ***g*** for 70 min in a Beckman SW60Ti rotor. Different fractions were withdrawn, diluted in TACM buffer and centrifuged at

504 000 *g* for 30 min in a Beckman 70Ti rotor (Beckman, Fullerton, CA, USA). The pellets were suspended in 0·2 M phosphate buffer pH 7·0. Purification steps were monitored by uranyl acetate negative staining and electron microscopy (Philips CM 10: Eindhoven, the Netherlands), and by DAS-ELISA.

Antibodies

The rabbit antiserum was that described by Garcia *et al.* (1997). To produce mabs, anaesthetized six-week-old BALB/c mice were injected in the spleen with partially purified virus resuspended in 100 μL of sterile saline (day 0), using a surgical opening in the peritoneum. At 3-week intervals for 2 months, further virus preparations resuspended in 200 μL of saline were injected intravenously. Four days after the last injection, the serum of each mouse was titrated against the virus by TAS-ELISA; the spleen of the mouse with the highest titre was removed and the cells fused with cells of the line P3/X63-Ag8·653 using PEG (MW 1500–1700, Sigma, St Louis, MO, USA) (Perotto *et al.*, 1992). Spent media from the primary cultures were screened by TAS-ELISA, and positive colonies were cloned three times at limit dilution and then grown as ascites tumours in pristane-primed mice. The isotype of the mabs selected was determined in Ouchterlony tests.

ELISA protocols

The protocols given below are those finally arrived at. In the Results, data are given on some variations, all tested with the homologous isolate, CRSV-4. One variant having a notable effect was the type of plate washing. Two automatic plate washers were used, W1 with pressurized jets and W2 employing gravity feed. Plates were also washed by hand using squirts from a wash bottle, emptying the plates, then tapping them sharply on the bench to remove all liquid. The number of washes was also varied to arrive at optimal conditions. A second variant was the incorporation of defatted milk at various stages, including its addition to the antigen during homogenization of the sample.

Optical density (OD) readings of all plates were taken at intervals, with a Biorad (Richmond, CA, USA) 3550 Microplate Reader (measurement 405 nm, reference 490 nm). The plate reader was zeroed on border wells filled with substrate solution. The data reported are those recorded when differences between the ratio of infected (I) to healthy (H) readings was greatest.

DAS-ELISA

The immunoglobulin (Ig)G and conjugate derived from the rabbit antiserum were as described by Garcia *et al.* (1997). The plates (Microlon 96 W high binding; Greiner, Frickenhausen, Germany) were coated with IgG at 0·5 μg mL^{-1} in standard coating buffer and incubated at 37°C for 4 h. The plates were washed with PBS-T five times by hand, in this and all washing steps. Healthy or infected citrus leaves were placed in a polythene bag with 9 parts (w/v) of extraction buffer (PBS-T containing 2% PVP and 2% defatted milk powder) and homogenized with a ball-bearing press. After overnight incubation with the homogenate at 4°C, the plates were washed and conjugate added, diluted 1/1000 in extraction buffer. Further incubation for 4 h at 37°C was followed by washing and addition of substrate at 1 mg mL^{-1}.

TAS-ELISA

The method of Thomas *et al.* (1986) was used as follows. Wells were coated with crude antiserum diluted 1/9000 in coating buffer and the plates incubated for 4 h at 37°C. Plate washing and preparation and incubation of antigens were performed as for DAS-ELISA. Ascites fluid containing the mabs, diluted 1/32 000 in extraction buffer, was added and the plates were incubated for 90 min at 37°C. Alkaline phosphatase conjugates (Sigma) of antimouse IgG (whole molecule, from rabbit) and antimouse IgM (μ-chain-specific, from goat), diluted 1/20 000 in extraction buffer, were added and incubated for 90 min at 37°C. Substrate (1 mg mL^{-1}) was added and the OD was read after two or more hours.

Western blots

These were prepared by standard procedures (Sambrook *et al.*, 1989) using PVDF membranes (Polyscreen, NEN, Life Science Products, Boston, USA). The antigens were precipitated from leaf homogenates using trichloracetic acid (Wu & Wang, 1984). IgG from the rabbit antiserum was further cross-absorbed with healthy TCA-precipitated material and used at 0·9 μg mL^{-1}. The mabs were used at 1/1000 dilution of the ascites fluid.

Results

Purification

The method described, starting from *G. globosa,* gave virus yields about 1·5 times greater and somewhat cleaner than when starting from *C. quinoa*. However, after density gradient centrifugation, no clearly defined light-scattering bands were formed, but a virus-rich zone 18–26 mm from the base of the tube was observed. Systemic infections in *G. globosa* had the additional advantage of providing a source of virus over an extended period.

DAS-ELISA

The protocol described in the materials and methods section improved the sensitivity of the DAS-ELISA four-fold over that reported by Garcia *et al.* (1997). In the direct comparison of the methods (Table 2: plate A

Table 2 Comparison of five DAS-ELISA and two TAS-ELISA protocols using the same CPsV-infected citrus leaf extract diluted in fivefold steps from 1/10 to 1/31250, using healthy citrus leaf extract at 1/10 as diluent. A to G are results for seven plates prepared at the same time. A and F refer to the optimized DAS and TAS, respectively. The others are as indicated. W1 and W2 are the two plate washers. The OD figures refer to mean optical densities (standard deviations in brackets) for 6 wells (infected) and 20 wells (healthy)

	A DAS Milk plus Wash 5×hand Reading 1 h		B DAS Milk plus Wash 3×W1 Reading 1 h		C DAS Milk plus Wash 3×W2 Reading 1 h		D DAS Milk minus Wash 3×W1 Reading 3 h		E DAS Milk minus Wash 3×W2 Reading 3 h		F TAS IgG Milk plus Wash 5×hand Reading 3 h		G TAS IgM Milk plus Wash 5×hand Reading 3 h	
Dilution	OD	I/H	OD	I/H	OD	I/H	OD	I/H	OD	I/H	OD	I/H	OD	I/H
1/10	1·829 (0·31)	108	2·590 (0·04)	25	2·371 (0·12)	20	1·179 (0·03)	21	1·395 (0·16)	18	2·648 (0·04)	441	2·537 (0·05)	634
1/50	1·897 (0·25)	112	2·229 (0·09)	22	2·313 (0·09)	19	0·935 (0·02)	17	1·049 (0·17)	13	2·431 (0·03)	405	1·996 (0·17)	499
1/250	0·895 (0·05)	53	1·226 (0·07)	12	1·253 (0·09)	11	0·351 (0·02)	6·4	0·407 (0·04)	5	1·672 (0·12)	279	0·921 (0·14)	230
1/1250	0·249 (0·04)	15	0·454 (0·05)	4·5	0·438 (0·03)	3·7	0·128 (0·01)	2·3	0·130 (0·05)	1·6	0·471 (0·07)	79	0·233 (0·02)	58
1/6250	0·066 (0·007)	4	0·153 (0·010)	1·5	0·178 (0·010)	1·5	0·057 (0·012)	1	0·093 (0·009)	1·2	0·115 (0·009)	19	0·053 (0·007)	13
1/31250	0·027 (0·004)	1·6	0·155 (0·039)	1·5	0·141 (0·011)	1·2	0·053 (0·013)	0·9	0·089 (0·018)	1·1	0·036 (0·003)	6	0·022 (0·003)	6
Healthy 1/10	0·017 (0·009)		0·102 (0·030)		0·119 (0·028)		0·055 (0·013)		0·079 (0·015)		0·006 (0·002)		0·004 (0·004)	

compared with plates D and E), however, the difference was even greater. The homologous antigen in infected greenhouse citrus was detected down to a tissue dilution of 1/6250 (plate A, I/H ratio of 4). I/H-values of over 100 were obtained at tissue dilutions from 1/10 to 1/100 in this and in many other tests. All isolates that were tested by DAS-ELISA (Table 1) came up positive, so the correlation between indexing and these results was exact.

Virus in leaf material dried over silica or calcium chloride and stored at 4°C could be detected by DAS-ELISA over a period of at least 2 months, but air-drying of material led to loss of antigenicity.

Experiments to optimize the protocol showed that the plate-washing procedure was important for obtaining consistently low OD values for healthy preparations, without significantly lowering values for infected samples. They also showed that adding defatted milk to the antigen and the conjugate gave improvement, especially if combined with thorough washing.

Comparing the improved protocol (Table 2, plate A) with standard machine plate washing with either of the plate washers (Table 2, plates B and C), I/H ratios of approximately four times higher for all tissue dilutions up to 1/1250 were obtained. A cycle of five machine washes instead of the standard three washes did not significantly improve these I/H ratios. These results were confirmed in an experiment where infected or healthy sap extracts were diluted 1/10, 1/100 or 1/1000 and assayed in plates washed three or five times, by machine or by hand. The results gave I/H-values, for the three dilutions, of 898, 699 and 115 for five hand washes, 60, 50 and 11 for three hand washes, 14, 13 and 5 for five machine washes, and 11, 10 and 5 for three machine washes. Additional hand washes lowered the I/H-values, probably because they began to remove even some specifically bound reactants.

Adding milk also improved the I/H ratios, as seen by comparing, in Table 2, plates B and C (milk plus) with D and E (milk minus). These data were obtained using three standard machine washes but further experiments showed that, with five washes by hand, the advantage of incorporating milk became considerably greater.

Mabs

Two cell lines secreting mabs to CPsV were selected. One mab was an IgG1 and the other an IgM. Ascites fluid titres, determined in TAS-ELISA as the dilution giving half the maximum (plateau) absorbance values, were about 1/256 000 for both the IgG and the IgM.

TAS-ELISA

As with the DAS-ELISA, preliminary experiments allowed optimization of the system, and showed that the effects of plate washing and of including defatted milk in the antigen, mab and conjugate preparations were similarly beneficial. Substituting ovalbumin, gelatin or BSA for milk gave I/H ratios less than one third as high (data not shown).

When the same serially diluted extracts of the

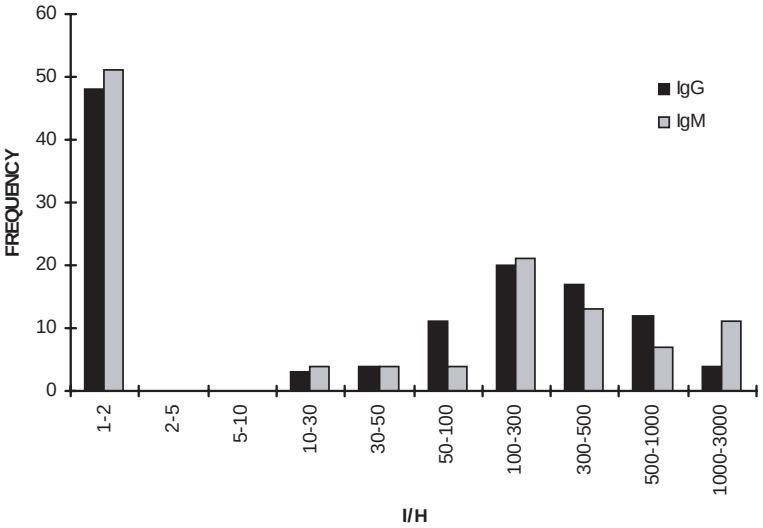

Figure 1 Frequencies of different I/H-values obtained with sample dilutions of 1/10 using the IgG and IgM mabs in TAS-ELISAs. A total of 119 citrus leaf samples from field and greenhouse are represented. Each sample was tested with each mab. Note that no sample gave I/H-values in the ranges 2–10, an indication that the results were always either clearly positive or clearly negative.

homologous virus isolate were compared by DAS and TAS-ELISA, the TAS formats (with both the IgG and the IgM mabs) were at least five times more sensitive than the best DAS system (Table 2) and gave I/H-values above 3, with both mabs, at a tissue dilution of 1/31 250 (Table 2, plates F and G). This was mainly because TAS H-values were consistently low (0·001–0·006).

When five CPsV isolates (CRSV-4, RS-SOR, P216 m, P213 and P215 m) were compared by optimized DAS, IgG-TAS and IgM-TAS, at a tissue dilution of 1/10, the I/H-values for each isolate were 8–13 times higher in the TAS-ELISAs than in the DAS-ELISA (data not shown).

Screening 119 samples (including field samples taken in winter, and the glasshouse material listed in Table 1) at a tissue dilution of 1/10, using each of the mabs in TAS-ELISA, I/H-values of between 12 and 3000 (clearly positive) or clustering at 2 or less (clearly negative) were obtained, with no values in the range 3–11 inclusive (Fig. 1).

The IgG mab detected all isolates tested (Table 1), indicating that the epitope recognized is present in a wide range of isolates and again confirming the correlation with biological indexing. In parallel tests the IgM mab was equally effective except that it did not react with isolates IAM-320X from southern Italy, and P-121 and RS-105 from Spain. Where the IgM gave positive results, the I/H-values were as high as with the IgG, and use of this mab raised no problems.

Western blots

The rabbit antiserum reacted strongly in Western blots, giving a band corresponding to the coat protein of CPsV (48 kDa: Barthe *et al*., 1998). The IgG mab reacted to give a similar band but only at an antigen concentration four times higher. The IgM mab did not react even then. The results suggest that the denatured antigen made a poor target for the mabs.

Discussion

The results show that TAS-ELISA employing the IgG mab is a powerful tool for detecting CPsV, and should be useful in diagnostic and quarantine programmes. The assay promises to be sensitive enough to detect the virus in low concentration, at unfavourable seasons of the year, or in composite samples that include some healthy material. The fact that it has detected all available isolates suggests that this mab may react with an epitope displayed by a wide range of variants. The IgM mab did not detect three isolates, but it should be useful, with other mabs such as those of Djelouah *et al.* (1999), in differentiating strains of CPsV.

The DAS-ELISA is highly sensitive in its new form but less sensitive than the TAS-ELISA. It will still be useful because it has a good chance of detecting all CPsV isolates.

The experiments showed that major factors in improving the sensitivity of both the DAS and TAS-ELISAs were incorporation of defatted milk into the extraction buffer and all subsequent reagents (except the substrate), and thorough washing of the plates. The latter was important in itself but became especially critical when milk was used.

Defatted milk has rarely been used in the extraction buffer for preparing ELISA samples, and no case has been found where it was also added to the conjugate and mab dilution buffers. Ragetli *et al.* (1982) used it when purifying *Little cherry virus* from cherry leaves but their goal was virus purification, not ELISA. Habili *et al.* (1997) used it at 0·5% in the extraction buffer for ELISA detection of *Grapevine leafroll-associated virus 1*, but without giving reasons.

Plate washing was carefully examined because impressive improvements were obtained by hand washing as opposed to machine washing, although usually no distinction is made in the literature between the two. Generally a regime of three washes is recommended

(for example see Converse & Martin, 1990; Torrance, 1992; Fox, 1993; Van Regenmortel & Dubs, 1993) but Tijssen (1985) notes that 'as many as 6 washes may be necessary instead of the usual 2 or 3 advocated in standard procedures for the complete removal of loosely bound immunoreactants' (p. 298). Copeland (1998) emphasized that flaws in the performance of mechanical washers are a common source of elevated background absorbance values, and that even where a plate washer can aspirate the contents of wells, it is better to empty them by hand. Beumer *et al.* (1992) also noted many shortcomings in mechanical plate washers and improvements that might be made to them. The present data suggest that a cycle of three machine washes allows a significant fraction of nonspecific reactants to remain in the wells and that two further machine washes give little improvement. In contrast, three hand washes already give good results, which continue to improve with up to five washes.

Comparing the protocols reported here with those published for detection of five other widely different viruses, in both woody and herbaceous hosts, it was found (M. Gangemi and D. Alioto, unpublished results) that these protocols give significant I/H increases, similar to those obtained with CPsV, in all cases. This experience suggests that ELISA may have become so routine that optimization is no longer always seriously considered, and that a fresh look at some of the variables could be beneficial.

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