

IMMUNODIAGNOSIS OF *CUCURBIT YELLOW STUNTING DISORDER VIRUS* USING POLYCLONAL ANTIBODIES DEVELOPED AGAINST RECOMBINANT COAT PROTEIN

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SUMMARY

The coat protein (CP) gene of *Cucurbit yellow stunting disorder virus* (CYSDV) was amplified by reverse transcription – polymerase chain reaction (RT-PCR). The amplicon was cloned in pGEM-T, sequenced and subcloned into a bacterial expression vector (pQE-31). The recombinant CYSDV CP was expressed as a fusion protein with an N-terminal hexa-histidine tag, purified by affinity chromatography yielding 8 mg native protein per liter of bacterial culture, and used as an antigen to produce CYSDV CP antiserum in a rabbit. The resulting antiserum was successfully assayed in tissue blot immunoassay (TBIA), dot blot immunoassay (DBIA), indirect ELISA and DAS-ELISA, with a titer of about 10^3 for all methods. TBIA was very specific and showed the virus localization in the phloem tissue and is recommended for large-scale surveys.

Key words: CYSDV, recombinant coat protein, polyclonal antibodies, ELISA, TBIA.

INTRODUCTION

Virus diseases of cucurbits cause important economic losses throughout the world. More than 35 viruses have been reported to infect cucurbits (Provvidenti, 1996). In 1995, a review article about whitefly-transmitted cucurbit viruses stated that *Cucurbit yellow stunting disorder virus* (CYSDV), belonging to the family *Closteroviridae* and genus *Crinivirus*, was limited in distribution to the Middle East: United Arab Emirates, Syria, Turkey, Jordan, Egypt and Israel (Duffus, 1995). Later on, CYSDV spread to Mediterranean Europe where reports from Spain indicated that it has progressively replaced *Beet pseudo yellows virus* (BPYV), which was predominant in the Almeria region of Southern Spain (Celix *et al.*, 1996); this change in virus incidence has been associated with the increase in *Bemisia tabaci* populations (Jorda-Gutierrez *et al.*, 1993; Berdiales *et al.*, 1999). More recently CYSDV was reported in Portugal (Louro

et al., 2000) and Morocco (Desbiez *et al.*, 2000), and has crossed the Atlantic to North America (Kao *et al.*, 2000). The virus symptoms start as interveinal mottle on older leaves and develop into severe yellowing as the leaves grow older; leaves remain turgid and become brittle. Symptoms spread upward but never reach the youngest four to five leaves. In Lebanon, severe CYSDV infections of greenhouse-grown cucumbers cause great economic losses and result in approximately 50% yield loss (Abou-Jawdah *et al.*, 2000b).

CYSDV has long flexuous particles, 750-800 nm in length (Celix *et al.*, 1996; Liu *et al.* 2000), and a bi-partite genome. The coat protein of 28.5 kDa is coded by RNA 2, which is 7,281 nucleotides long and also codes for other proteins including the coat protein minor (CPm, 53 kDa) and the heat shock protein 70 homologue (HSP70h, 62kDa) (Livieratos and Coutts, 2002). In addition to CYSDV, two other viruses in the family *Closteroviridae* attack cucurbits: *Beet pseudo yellows virus* (BPYV) (genus *Closterovirus*) and *Lettuce infectious yellows virus* (LIYV) (genus *Crinivirus*). So far, laboratory diagnostic techniques have relied mainly on polymerase chain reactions (RT-PCR) using specific primers (Celix *et al.*, 1996). Other techniques such as nucleic acid hybridization and dsRNA analysis have also been described (Tian *et al.*, 1996). However, only one report described a serological detection method using indirect ELISA (Livieratos *et al.*, 1999). Due to the phloem limitation of these closteroviruses and their overall low titer in infected plants, it is difficult to purify sufficient quantities of their filamentous particles for production of high quality antibodies. Recombinant DNA technology was successfully used to overcome these problems and allowed the production of several specific antibodies for other closteroviruses (Agranovsky *et al.*, 1994; Klaassen *et al.*, 1994; Nikolaeva *et al.*, 1995; Hoyer *et al.*, 1996; Ling *et al.*, 1997; Karasev *et al.*, 1998; Livieratos *et al.*, 1999; Ling *et al.*, 2000). Cucurbit viruses constitute a complex and dynamically changing pathosystem (Nameth *et al.*, 1986). Therefore, for effective management of cucurbit virus diseases, periodical surveys are required to assess the relative importance of each virus. Serological tests, such as several variants of ELISA, are still considered the most appropriate techniques for screening large numbers of samples.

In this paper, we report the production of a high titer

CYSDV antiserum and its use in three serological assays: tissue blot immunoassay (TBIA), dot blot immunoassay (DBIA), and two variants of ELISA. TBIA is recommended in view of its simplicity, sensitivity, rapidity and the possibility to conduct the test in any laboratory with the minimum of facilities.

MATERIALS AND METHODS

Plant material, RT-PCR and cloning. Cucumber leaves infected with CYSDV were collected from a greenhouse in Jieh, Lebanon. Total RNAs were extracted from mid-vein tissues using TRI reagent (Molecular Research Center, USA) according to manufacturer's instructions, and were used as templates for RT-PCR. A pair of primers was designed to amplify the full-length (750 bp) of the coat protein gene (Table 1), based on the coat protein gene sequence published in the GenBank. Aliquots (2 µl) of the total RNA extracts were used in an RT-PCR reaction mixture (20 µl) containing 0.5 µM of CYSDV CP specific primers. The "Reverse-it One-Step RT-PCR Kit – Ready Mix Version" (Abgene, U.K), which permits the reverse transcription and polymerase chain reactions to be done in one step, was used according to the manufacturer's instructions. The RT-PCR reactions consisted of one cycle at 50°C for 45 min, one cycle at 94°C for 2 min, 40 cycles at 94°C for 30 s, 53°C for 90 s and 72°C for 90 s, and a final extension step at 72°C for 7 min. The amplicons were analyzed by electrophoresis in 1.2% agarose gel in 0.5x TAE buffer. The PCR products were then cloned into pGEM-T (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 cells. The nucleotide sequence was determined on both strands, at the University of Wisconsin Biotechnology Center, with an automated DNA sequencer. The resulting nucleotide and amino acid sequences were compared to the published complete CP genes of CYSDV (NCBI, AJ243000) and other closteroviruses, using the BLASTN 2.2.3 program (Altschul *et al.*, 1997).

Table 1. Specific primers designed for amplification of CYSDV coat protein gene.

Primer	Nucleotide sequence ^a
Forward - P1	5' AATAGCATGCAATGGCGAGTTCGAGTGAGAA 3'
Reverse - P2	5' AATTCTGCAGTCAATTACCACAGCCACCTG 3'

^a Underlined are the restrictions sites for *Sph*I and *Pst*I in P1 and P2 respectively.

Protein expression and antiserum production. The CYSDV CP gene was digested from the recombinant pGEM-T using *Sph*I and *Pst*I, and the 750 bp fragment

was gel-purified using the "QIAquick gel extraction kit" (Qiagen, Germany) and subcloned into the protein expression vector pQE-31 (Qiagen, Germany), which tags the expressed protein with N-terminal hexa-histidine (6xHis tag), allowing easier purification by affinity chromatography. The construct was, then, transformed into *E. coli* M15 cells. Cultures of the transformed M15 cells were grown to an optical density $A_{600nm} = 0.6$, and were, then, induced with 1mM isopropyl-thio-β-D-galactopyranoside (IPTG) for 4 h at 31°C. The bacterially expressed protein was purified to near homogeneity from cell lysates on a nickel-charged nitrotri-acetic acid (Ni-NTA) resin (Qiagen, Germany) following the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%), using the discontinuous Tris-glycine buffer system (Laemmli, 1970) and subsequent staining with Coomassie Brilliant Blue G-250, were performed to prove the identity and to assess the purity of the produced fusion protein. The purified protein was used to immunize a rabbit. The first injection was intramuscular, using 1 mg of recombinant CYSDV CP in 1 ml containing 1:1 (v/v) Freund's complete adjuvant (FCA), and was followed by four sub-epidermal injections at weekly intervals, using Freund's incomplete adjuvant (FIA) containing 1 mg recombinant protein. The rabbit was bled 10 days after the last injection and the antibodies were purified as previously described (Clark and Adams, 1977). Booster injections were administered monthly using 0.3 mg of CYSDV CP in FIA, and blood was collected 10 days later.

Western blotting. Proteins extracted from five CYSDV-infected cucumber plants and healthy plants using PBS in a 1:5 (w/v) ratio, along with affinity-purified recombinant CYSDV CP and prestained protein molecular weight standards (Prestained SDS-PAGE standards-Broad range, Bio-Rad, Italy), were separated by 10% SDS-PAGE. Electrophoresis of the separated proteins onto nitrocellulose membranes was done using the "Mini Trans-Blot Electrophoretic Transfer Cell" (Bio-Rad, Italy) at 100V for 90 min at 4°C. Membranes were blocked with 0.1% polyvinyl alcohol (PVA) for 1 min at room temperature and then incubated for 1 h with the purified antibody (1:1,000), followed by 1 h incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, Germany) (1:10,000). Washing with PBS-Tween 20 (PBS-T) was repeated three times following each step. Membranes were, then, incubated for 20 min with freshly prepared nitroblue tetrazolium (NBT, 0.35 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.18 mg/ml) in 0.1 M Tris buffer, pH 9.5, containing 0.1 M NaCl and 0.1 M MgCl₂.

Tissue Blot (TBIA) and Dot Blot (DBIA) immunoassays. For TBIA, the petioles of over hundred CYSDV-infected and healthy cucumber and melon

plants were cross-sectioned and pressed on Hybond nylon membranes (Amersham Life Sciences, Lebanon) or nitrocellulose membranes for few seconds. Nylon membranes were blocked for 1 h with PBS-T buffer containing 1% BSA and 5% skimmed milk, whereas nitrocellulose membranes were blocked with PVA. The membranes were incubated with the primary antibody, as described above, using three concentrations of the purified antibody: 1:1,000, 1:2,000, 1:5,000, and three concentrations of crude antiserum, 1:5,000, 1:10,000 and 1:15,000. Membranes were then incubated with the secondary antibody and then NBT/BCIP, as described for western blotting. The dried membranes were examined under the stereoscope for color development in the phloem cells where the virus is located.

For DBIA, dried and fresh tissues from CYSDV-infected and from healthy cucumber and melon plants were ground (1:5 w/v, on fresh weight basis) in phosphate buffer ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), pH 9.7, containing 0.2% DIECA. Samples of 3 μl were blotted on nylon membranes and nitrocellulose membranes and left to air dry. Also, 5, 10, 25, 50 and 100 ng of expressed proteins were blotted on the same membranes. The membranes were, then, processed as for TBIA, except that only two concentrations of purified antibodies were used: 1:1,000 and 1:2,000, and one crude antiserum concentration, 1:10,000.

ELISA. Indirect plate-trapped ELISA and DAS-ELISA were tested. For indirect ELISA, three extraction buffers were compared: ELISA carbonate coating buffer (pH 9.6, containing 0.2% DIECA), PBS-T (pH 7.4, containing 0.1% sodium sulfite) and phosphate buffer (pH 9.7, containing 0.2% DIECA). ELISA polystyrene plates were used with or without overnight coating with poly-L-lysine. Fresh cucumber leaf tissue was triturated in the extraction buffers (1:10 w/v) and ELISA plates were coated with 100 μl extracts and incubated overnight at either 4°C or 37°C. Also, 2, 5, 10 and 50 ng of CYSDV recombinant CP, diluted in PBS-T containing 0.1% sodium sulfite, were used in the test. The wells were then blocked for 1 h at 37°C with 200 μl /well PBS-T containing 1% BSA and 5% skimmed milk. Purified antiserum (IgG 1 mg/ml) was added at a concentration of either 1:50, 1:100, 1:500, 1:1,000 or 1:2,000 in PBS and incubated at room temperature for 3 h. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, Germany), diluted to 1:10,000 in ELISA conjugate buffer, was added and incubated for 2 h at 37°C. Three washings with PBS-T were done after each step. Finally, the substrate (1 mg ml^{-1} *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) was added and incubated at room temperature or 37°C for 1-3 h or overnight at 4°C. The color reaction was monitored by measuring absorbance at 405 nm, using a microplate ELISA reader.

DAS-ELISA was done as described by Clarks and

Adams (1977). In this test, coating the plates with poly-L-lysine was also tried. The purified antiserum was used at a concentration of 1:1,000 and 1:2,000, and the prepared alkaline-phosphatase-conjugated CYSDV specific IgG was used at a concentration of 1:450, 1:750, 1:1,500 or 1:3,000.

A reaction was considered positive when the mean absorbance value for a sample was greater than twice the mean value of healthy controls. All serological tests were repeated at least three times.

RESULTS

RT-PCR and cloning. Agarose gel electrophoresis, following RT-PCR of symptomatic samples, revealed a band of about 750 bp, which is the expected size of CYSDV CP gene (Fig. 1). No bands were amplified in assays of negative healthy samples. Amplicons with very sharp bands were ligated to pGEM-T, which was used to transform *E. coli*. The inserts in the clones were sequenced and the resulting nucleotide and amino acid sequences proved the identity of the CYSDV CP sequence, with more than 99% similarity to the published CP gene and amino acid sequences.

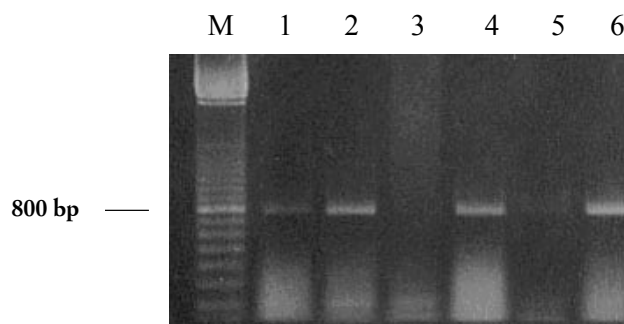


Fig. 1. Agarose gel electrophoresis of RT-PCR amplified products obtained from total RNA extracts of CYSDV-infected and healthy cucumber samples, using two specific primers that amplify the coat protein of CYSDV. M = 100 bp ladder. 1, 2, 3, 4 and 6: cucumber samples showing yellowing symptoms, collected from different locations. 5: healthy control.

Protein expression and antiserum production. After subcloning the CP gene into the expression vector pQE-31, the protein was expressed in *E. coli* and purified by affinity chromatography. SDS-PAGE analysis of the expressed proteins revealed one major protein band with a molecular weight of approximately 35 kDa. The yield of recombinant CYSDV CP was approximately 8 mg per liter of bacterial culture. Non-transformed bacteria included in the protein expression run produced a faint band of about 28 kDa (Fig. 2). The antiserum produced against CYSDV CP was purified, adjusted to a final concentration of 1 mg/ml ($A_{280\text{nm}} = 1.4$) and used in serological tests.

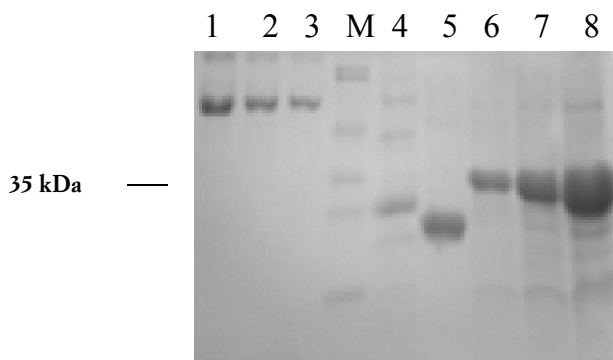


Fig. 2. Discontinuous SDS-PAGE of recombinant CYSDV CP in 10% polyacrylamide gel, stained with Coomassie Brilliant Blue. 1, 2, and 3: 8, 4 and 2μg BSA, respectively. M: broad range molecular weight protein standards. 4: non-transformed bacteria. 5: positive control, DHFR protein. 6, 7 and 8: affinity purified recombinant CYSDV CP, 3rd, 2nd and 1st elution, respectively. Loading volume: 10 μl/well.

Western blotting. Western blot analysis of the purified bacterially expressed protein with the antiserum revealed one major specific protein band of about 35 kDa and one very faint band of approximately 80 kDa. This same reaction pattern was observed in transferred bands from extracts of CYSDV-infected plants, but with slightly lower molecular weights, due presumably to the absence of the 6xHis tag in the viral CP isolated from plants (Fig. 3). These results suggest that the 35 kDa band contains the intact CP of CYSDV and that the other band is probably a polymer of this protein. No reaction was observed with extracts of non-transformed bacteria and healthy plant controls.

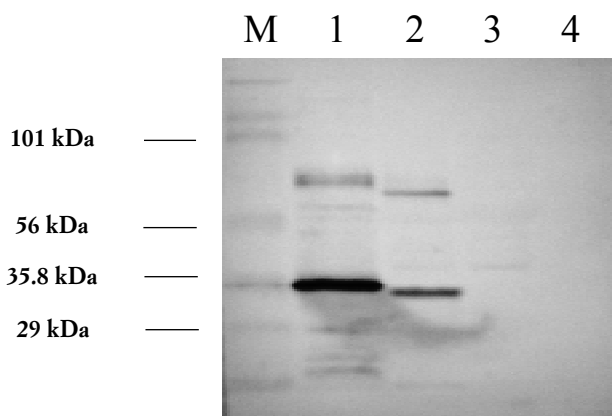


Fig. 3. Western blot analysis following electrophoresis in SDS-PAGE, electroblotting and detection with purified antibodies (1:1,000) against recombinant CYSDV CP. M: prestained broad range molecular weight protein standards, 1: recombinant CYSDV CP (0.5 μg), 2: CYSDV infected plant, 3: non-transformed bacteria, 4: healthy plant.

TBIA and DBIA. In TBIA, the purified serum was able specifically to detect CYSDV particles in tissue blots, inside the phloem cells of infected plants, on ny-

lon membranes only. The reaction was still strong even at an antibody dilution of 1:5,000 or crude serum dilution of 1:15,000. No color development was observed in prints of healthy non-infected tissues (Fig. 4).

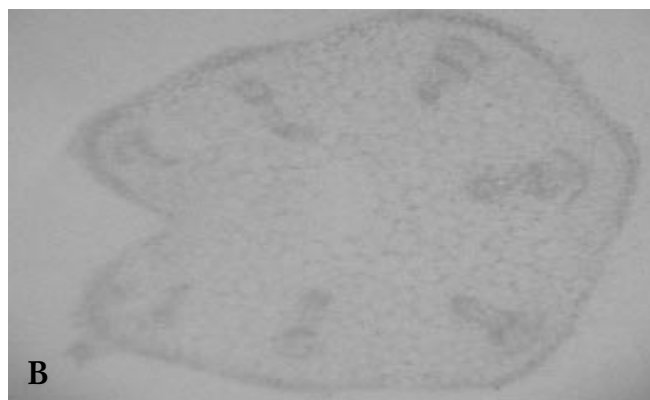
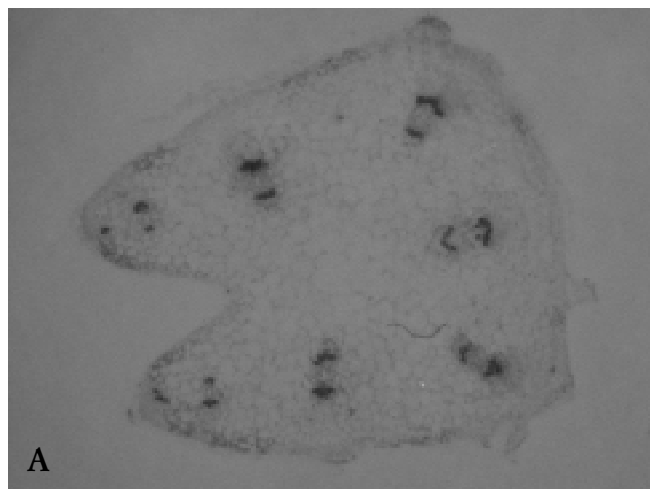


Fig. 4. TBIA of CYSDV infected plant, **A**; and healthy plant, **B**. The primary antibodies were the purified antibodies against CYSDV CP (1:2000) and the secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit IgGs (1:10,000).

In DBIA, the purified serum gave a strong positive reaction in dots of infected plant tissues and no color development in dots of healthy plant tissues, on both nylon and nitrocellulose membranes, and with both pure antibody dilutions (1:1,000 and 1:2,000) and crude antibody dilutions (1:5,000 and 1:10,000) (Fig. 5 and 6). In dots of recombinant CYSDV CP, the reaction intensity decreased with the decreasing amount of protein, and was still strong with 1 ng recombinant CP when a pure IgG dilution of 1:1,000 was used, and with only 5 ng when a dilution of 1:2,000 was used (Fig. 5). Fresh samples gave stronger reactions than dried samples, and crude antiserum was able to detect infected samples with low virus concentrations, better than the purified antiserum (Fig. 5 and 6).

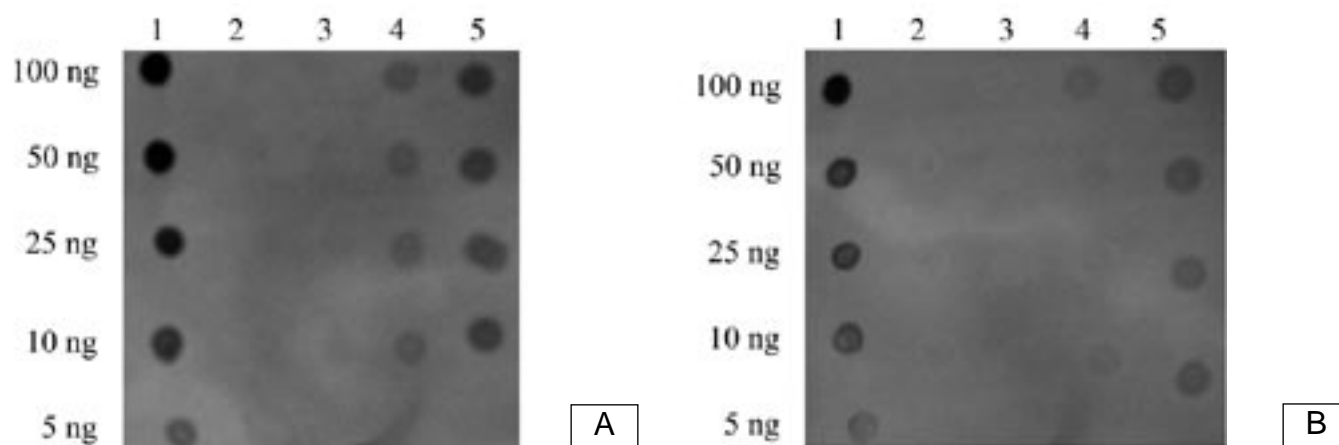


Fig. 5. DBIA of dried CYSDV infected plants and recombinant CP protein, on nylon membranes, using **A)** crude antiserum (1:10,000) and **B)** purified antibodies (1:2,000). 1: CYSDV recombinant CP, concentrations ranging from 100 to 5 ng; 2 and 3: 3 ml extracts from healthy controls; 4 and 5: 3 ml extracts from four CYSDV infected samples (extracted in phosphate buffer). The secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit IgGs (1:10,000).

ELISA. In indirect plate-trapped ELISA, twenty min incubation with the substrate was enough to produce a strong reaction with extracts of CYSDV-infected plants when using an antibody dilution of 1:1,000, and an antigen incubation at 4°C. In two of the buffers used, readings at $A_{405\text{nm}}$ ranged between 5 and 18 times higher than those of their respective negative controls. The highest readings were observed with PBS-T buffer (pH 7.4, containing 0.1% sodium sulphite) and the lowest with phosphate buffer (pH 9.7 containing 0.2% DIECA). The system was able to detect the recombinant proteins in quantities as little as 2 ng. The $A_{405\text{nm}}$ readings for the recombinant proteins were enhanced when the plate was pre-coated with poly-L-lysine, especially when the samples were incubated at 37°C (Tables 2 and 3).

DISCUSSION

Yellowing virus diseases of cucurbits are becoming increasingly important in many geographical regions (Wisler *et al.*, 1998). They may be transmitted by whiteflies (LIYV, BPYV and CYSDV) (Duffus, 1965; Duffus *et al.*, 1986; Brown and Nelson, 1986; Hassan and Duffus, 1991) or by aphids [*Cucurbit aphid-borne yellows virus* (CABYV)] (Lecoq *et al.*, 1992).

The rapid spread of CYSDV from the Middle East to Mediterranean Europe, Africa and North America, makes it one of the most important cucurbit viruses. In greenhouse-grown cucurbits in Spain and Lebanon, CYSDV is rapidly becoming the predominant virus (Livieratos *et al.*, 1999).

These changes of relative importance of viruses have

Table 2. Indirect ELISA using purified antibodies against recombinant CYSDV CP (1:1,000) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000). Overnight antigen incubation at 37°C.

	Plate untreated			Plate pretreated with poly-L-lysine		
	Buffer A ^a	Buffer B ^b	Buffer C ^c	Buffer A	Buffer B	Buffer C
Positive	0.350	1.270	1.250	0.580	1.750	0.730
Negative	0.130	0.160	0.135	0.130	0.170	0.150
CYSDV CP 0.5 µg/ml		1.500			> 3.000	
CYSDV CP 0.1 µg/ml		0.800			1.570	
CYSDV CP 0.05 µg/ml		0.400			1.120	
CYSDV CP 0.02 µg/ml		0.300			0.550	

^a Buffer A: Phosphate buffer, pH 9.7, containing 0.2% DIECA

^b Buffer B: PBS-T, pH 7.4, containing 0.1% sodium sulfite

^c Buffer C: ELISA carbonate coating buffer, pH 9.6, containing 0.2% DIECA

Table 3. Indirect ELISA using purified antibodies against recombinant CYSDV CP (1:1,000) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000). Overnight antigen incubation at 4°C.

	Plate untreated			Plate treated with poly-L-lysine		
	Buffer A ^a	Buffer B ^b	Buffer C ^c	Buffer A	Buffer B	Buffer C
Positive	1.250	2.250	1.420	0.490	1.130	0.950
Negative	0.115	0.125	0.125	0.230	0.230	0.195
CYSDV CP 0.5 µg/ml		1.400			1.840	
CYSDV CP 0.1 µg/ml		0.920			1.450	
CYSDV CP 0.05 µg/ml		0.450			0.760	
CYSDV CP 0.02 µg/ml		0.580			0.500	

^a Buffer A: Phosphate buffer, pH 9.7, containing 0.2% DIECA

^b Buffer B: PBS-T, pH 7.4, containing 0.1% sodium sulfite

^c Buffer C: ELISA carbonate coating buffer, pH 9.6, containing 0.2% DIECA

been previously reported for other cucurbit viruses and have been often associated with changes in vector species or strains.

The dynamic nature of cucurbit virus diseases necessitates periodical surveys to assess the relative importance of viruses in order to develop appropriate management strategies. Such surveys are best carried using serological tests. ELISA tests are available for most aphid-borne viruses: CABYV, *Zucchini yellow mosaic virus* (ZYMV), *Papaya ringspot virus* type Watermelon (PRSV-W), *Watermelon mosaic virus* (WMV) and *Cucumber mosaic virus* (CMV). However, no ELISA kits are commercially available for cucurbit viruses transmitted by whiteflies. Yellowing diseases of cucurbits show more or less similar symptoms under field conditions. Surveys conducted in Lebanon showed mixed infections by CABYV and CYSDV (Abou-Jawdah *et al.*, 2000a,b). Since most surveys are normally conducted using serological tests, CYSDV may not have been detected if RT-PCR was not specifically performed for its detection. The low titer of whitefly-transmitted cucurbit viruses in infected plants and the difficulty of their isolation in sufficient quantities and purity may have hindered the development of serological tests. Therefore, their diagnosis still relies heavily on nucleic acid-based techniques. Only one paper on the use of serological techniques to detect CYSDV has been published, (Livieratos *et al.*, 1999); but as in many other studies (Ling *et al.*, 2000; Kumari *et al.*, 2001), the polyclonal antibodies used were produced against the denatured recombinant coat proteins.

In this study, recombinant DNA technology was used to express CYSDV CP in bacterial cultures and to purify it in its native, non-denatured, form. An antiserum of high titer (about 10³) was produced. Western blot analysis showed that the antibody was specific to CYS-

DV CP. It gave a strong reaction with the recombinant CP and with extracts of CYSDV-infected plants, but it did not react either with proteins from healthy plants or with proteins from non-transformed bacterial cultures. When the serum was cross-adsorbed to the recombinant protein it no longer reacted in western blots, indicating the specificity of the antiserum. When it was adsorbed with another recombinant protein produced in the same manner (mouse dihydrofolate reductase, DHFR), the reaction was not altered. Amino acid-based alignment of the CYSDV CP with homologous proteins of other viruses revealed only limited amino acid similarities. The highest levels of similarity were with three closteroviruses, *Cucumber yellows virus*, *Sweet potato chlorotic stunt virus* (SPSCV) and LIYV (percentage identity: 40, 37 and 28 %, respectively), all members of the genus *Crinivirus*. Therefore, it would be interesting to test the specificity of the produced antiserum towards criniviruses.

Several serological assays were compared. In the standard DAS-ELISA, even though the purified polyclonal antibodies allowed differentiation between infected and healthy samples, the ratio of readings at A_{405nm} was only 2-3x (infected/healthy) and required a minimum of 3 h substrate incubation at 37°C.

On the other hand, indirect ELISA was highly sensitive. It gave high absorbance values within 30 min of substrate incubation. The ratio of A_{405nm} readings ranged between 5-18x (infected/healthy) depending on sample extraction buffer used and the temperature of antigen incubation. The best results were obtained with PBS-T buffer whether the overnight incubation of the antigen was at 4 or 37°C. If overnight sample incubation has to be done at room temperature or at 37°C, coating the microtiter plates with poly-L-lysine may improve the readings by about 1.5x. Indirect ELISA may

be the favored technique when determination of virus concentration is important, or when the laboratory is conducting ELISAs for other cucurbit viruses and the results need to be documented in the same manner for all viruses.

These results are in general agreement with those reported in the literature. Although several reports have appeared on the use of recombinant proteins to produce antisera, some of the antisera were used only in western blots (Hoyer *et al.*, 1996), others were successful only with indirect ELISA (Nikolaeva *et al.*, 1995; Livieratos *et al.*, 1999), but only a limited number were effective in DAS-ELISA (Varia *et al.*, 1996; Ling *et al.*, 2000). The frequently reported ineffectiveness of such antisera in DAS-ELISA was mainly attributed to their low ability to trap the virus particles or to high background reactions (false positives) obtained with healthy controls (Nikolaeva *et al.*, 1995; Ling *et al.*, 2000; Kumari *et al.*, 2001).

Dot blot immunoassays save time and antibodies, since the antibody solution can be reused several times. In our assays, the colorimetric detection method used allows detection of as little as 1 ng of virus protein and differentiates between healthy and infected samples. It can be used effectively to conduct surveys with large numbers of samples. Moreover, the use of a chemiluminescent detection method will give a higher sensitivity and will allow determination of virus concentration in plant tissue.

In TBIA, very clear precipitation spots in regions corresponding to the phloem tissue may be observed with slight magnifications (5-20x) allowing clear distinction between CYSDV-infected and non-infected samples, even at crude serum dilution of 1:10,000 to 1:15,000. In this manner, false positives may be identified easily because of the localization of infection and the corresponding precipitation pattern. Twenty-three cucumber and melon samples showing yellowing symptoms were collected from the field and tested by RT-PCR and TBIA. All samples that tested positive in RT-PCR were also positive in TBIA, and the same applies for the negative samples (data not presented). Therefore, TBIA is highly recommended for large-scale surveys of CYSDV and is especially well suited for use in developing countries. No sample preparation is required; the technique is very simple, does not require special skills, and is considered more economical than DAS-ELISA. However, when fresh samples are not available, DBIA would be recommended.

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