Cucurbit aphid-borne yellows virus Is Prevalent in Field-Grown Cucurbit Crops of Southeastern Spain

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ABSTRACT

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Despite the importance of field-grown cucurbits in Spain, only limited information is available about the impact of disease on their production. During the 2003 and 2004 growing seasons, systematic surveys were carried out in open field melon (*Cucumis melo*) and squash (*Cucurbita pepo*) crops of Murcia Province (Spain). The fields were chosen with no previous information regarding their sanitation status, and samples were taken from plants showing viruslike symptoms. Samples were analyzed using molecular hybridization to detect *Beet pseudo-yellows virus* (BPYV), *Cucurbit aphid-borne yellows virus* (CABYV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Melon necrotic spot virus* (MNSV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV). We collected 924 samples from 48 field plots. Out of these, almost 90% were infected by at least one of the viruses considered, usually CABYV, which was present in 83 and 66% of the melon and squash samples, respectively. In the case of melon, CYSDV, BPYV, and WMV followed CABYV in relative importance, with frequencies of around 20 to 30%, while in squash, CVYV and BPYV showed frequencies between 28 and 21%. The number of multiple infections was very high, 66 and 56% of the infected samples of melon and squash, respectively, being afflicted. CABYV was present in all multiple infections. The high incidence of CABYV in single and multiple infections suggests that this virus may well become an important threat for cucurbit crops in the region. Restriction fragment length polymorphism (RFLP) analysis revealed that CABYV isolates can be grouped into two genetic types, both of which seemed to be present during the 2003 epidemic episode, but only one of the types was found in 2004.

Additional keywords: aphid transmission, mixed infection, variability, whitefly transmission, yellowing disease

The major cultivated cucurbit species (melon, cucumber, squash, and watermelon) are important vegetable crops worldwide, especially in developing countries. Spain occupies fifth place in the world production of melon and is the most important producer in Europe. In Spain, melon follows tomato as the most cultivated vegetable crop (35,200 ha;

1,176,900 t/year) (12). Almost 75% of Spanish melon production is from field-grown crops, mainly in the southern and eastern areas of the country. Murcia Province, located in the southeastern part of the country, is one of the most intensive cucurbit cultivation areas, its melon production accounting for 19% of the total Spanish production (6,280 ha; 223,200 t/year) (Region de Murcia Digital).

Virus-induced diseases are a worldwide problem for cucurbit production and cause serious economic losses. Indeed, more than 35 different viruses have been isolated from cucurbits (29). Yellowing diseases have been observed in Spain for more than 20 years, but while these disorders were first attributed to nutritional deficiencies, the use of appropriate diagnostic tools revealed that they were mostly caused by

viruses. During the 1980s, yellowing diseases were associated with *Beet pseudo-yellows virus* (BPYV, genus *Crinivirus*) transmitted by *Trialeurodes vaporariorum* (17). However, during the 1990s, *T. vaporariorum* was progressively displaced by *Bemisia tabaci*, the vector for a new crinivirus, *Cucurbit yellow stunting disorder virus* (CYSDV) (5). Additionally, mosaic-inducing viruses, such as *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) and potyviruses *Watermelon mosaic virus* (WMV), *Papaya ringspot virus* (PRSV), and *Zucchini yellow mosaic virus* (ZYMV), have also become widespread in cucurbit crops in Spain (3,24). Emerging viruses such as *Cucumber vein yellowing virus* (CVYV, genus *Ipomovirus*) (26) and *Cucurbit aphid-borne yellows virus* (CABYV) (18) may become important problems.

CABYV is a member of the genus *Polerovirus*, family *Luteoviridae* (27). Its viral particles are isometric, approximately 25 nm in diameter, and encapsidate the CABYV genome, which consists of a single-stranded (ss) positive sense RNA molecule of 5.7 kb. It is transmitted in a persistent and circulative manner by at least two aphid species, *Aphis gossypii* and *Myzus persicae* (20). CABYV cannot be mechanically transmitted, and in nature, no other transmission method has been described for this virus. The host range of CABYV includes melon, cucumber, squash, and watermelon, in which it remains confined to the phloem. Other non-cucurbit species of high agronomic importance that are hosts for CABYV include lettuce (*Lactuca sativa*) and fodder beet (*Beta vulgaris*) (20).

In this study, we describe the results of a systematic survey carried out during two consecutive growing seasons (2003 and 2004) in melon and squash crops in the Murcia region of Spain. Out of the nine different viruses tested by molecular hybridization, CABYV was the most prevalent. We also conducted an analysis for the preliminary genetic characterization of Spanish CABYV isolates.

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MATERIALS AND METHODS

Surveys and sample collection. Surveys were performed in open field melon (*Cucumis melo*) and squash (*Cucurbita pepo*) crops of the Murcia Province during 2003 and 2004. In Murcia, melon and squash are grown during the summer and autumn seasons, respectively. Samples were obtained from 48 fields randomly distributed in a geographical area of approximately 300 km². Seven hundred melon and 224 squash samples from symptomatic plants from 36 melon and 12 squash fields were collected for virus detection. Each field was visited and sampled at least twice, before and at the beginning of the harvest. Incidence of viral symptoms was visually evaluated in each field by examining 300 to 900 plants following a W-shaped itinerary, and expressed as the percentage of the total plants. Viral symptoms observed included yellowing, mosaic, mottling, necrosis of the leaves and stems, vein clearing, and leaf lamina distortions. A maximum of 30 samples per field from symptomatic plants were collected. A sample consisted of two symptomatic leaves per plant.

Melon cultivars included Lavi, Primal (of "Galia" type), Cantasapo, Toledo, Nicolás, Olmedo (of "Piel de Sapo" type), Doral (of "Amarillo" type), and Branco de Ribatejo. Squash cultivars included Afrodite, Anissa, Mastil, Mikonos, Valiant, and Xsara.

Virus detection. Samples were analyzed to determine the presence of BPYV, CABYV, CMV, CVYV, CYSDV, PRSV, WMV, ZYMV, and also *Melon necrotic spot virus* (MNSV, genus *Carmovirus*). The samples were used immediately after collection for the detection of viruses by dot-blot hybridization (25). Samples from plants showing yellowing symptoms were additionally analyzed by tissue print hybridization (25) to detect the phloem-associated viruses BPYV, CABYV, and CYSDV. To detect viral RNAs, digoxigenin-11-UTP-labeled RNA probes were synthesized by in vitro transcription from the plasmids listed in Table 1. Plasmids to prepare probes for CYSDV, BPYV, CABYV, CMV, CVYV, and MNSV have been described elsewhere (8,13,18,25, 26,30). Plasmids to prepare probes for PRSV, WMV, and ZYMV were prepared specifically for this work from isolates PRSV-Z43, WMV-M116, and ZYMV-C16 (9) following standard procedures (31). Nucleotide sequences of cDNA inserts have been deposited in GenBank under accession numbers DQ645730 (PRSV-Z43), AF551334 (WMV-M116), and DQ645729 (ZYMV-C16). Plasmids were digested with the restriction enzymes indicated in Table 1, followed by ethanol precipitation. Transcription was performed with T7 or Sp6 polymerases (Table 1) and DIG-RNA labeling mix (Roche Diagnostics) according to manufacturer's instructions.

For dot-blot detection, 0.5 µl of total RNA extracts was spotted onto positively charged nylon membranes (Roche Diagnostics). Each extract was prepared as described in Célix et al. (5) from 0.2 g of leaf lamina tissue from the two leaves that constitute a sample. For tissue-print detection, the two leaf petioles of each sample were cut with a razor blade, and immediately after cutting, the cross-sections were blotted onto positively charged nylon membranes. The same protocol was used subsequently for both tissue prints and dot-blot. Briefly, membranes were prehybridized and hybridized at 68°C in standard buffer (50% formamide, 5× SSC, 0.1% N-laurylsarcosine, 0.02% sodium dodecyl sulfate [SDS], 2% blocking reagent [Roche Diagnostics]). Membranes were washed twice at room temperature in 2× SSC for 5 min, and twice at 65°C in 0.1× SSC for 15 min (25). Chemiluminescent detection was carried out using the reagents and protocols supplied by a DIG-labeling and detection kit (Roche Diagnostics). Positive and negative controls were included on each membrane and consisted of leaf tissue extracts and prints of petiole cross-sections (25) of healthy melon and squash plants, extracts of plants experimentally infected with CMV-Fny, CVYV-AILM, MNSV-Ma5, PRSV-Z43, WMV-M116, and ZYMV-C16, and extracts of healthy plants mixed with RNA of positive polarity of BPYV, CABYV, and CYSDV transcribed (31) from plasmids listed in Table 1. Under our conditions, cross-hybridization was never detected.

Amplification of the CABYV coat protein (CP) gene. Briefly, total RNA extracts were prepared from plant tissues as in Aranda et al. (4). Reverse transcriptase-polymerase chain reactions (RT-PCR) were carried out using oligonucleotides 5'-GAATACGGTCGCGGCTAGAAATC-3' (CE9) and 5'-CTATTCGGGTTCTGGACCTGGC-3' (CE10) (18), identical and complementary, respectively, to nucleotide sequences flanking the open reading frame 4 (CP) of the CABYV sequence published by Guilley et al. (15). The reverse transcription (Expand Reverse Transcriptase,

Roche Diagnostics) and PCR (Expand High Fidelity PCR enzyme mix, Roche Diagnostics) were performed according to manufacturer's instructions. A single DNA product of approximately 600 bp was obtained from the symptomatic samples, while RNA from healthy control plants gave no RT-PCR product.

Restriction fragment length polymorphism (RFLP) analyses of CABYV isolates. RFLP was chosen as a fast method for classifying the different CABYV isolates. DNA fragments of CABYV CP gene obtained by RT-PCR were digested with 10 different restriction enzymes: *Bam*HI, *Bcc*I, *Dra*III, *Eco*RI, *Fok*I, *Hind*III, *Kpn*I, *Sa* I, *Sal*I, and *Xba*I. After digestion, samples were fractionated by electrophoresis in 2% agarose gels and stained with ethidium bromide (31).

RESULTS

Incidence of CABYV. We analyzed 924 samples of symptomatic plants taken from 36 melon (summer) and 12 squash (autumn) commercial fields in Murcia. Almost 90% of the samples were infected by at least one of the viruses considered.

For melon, CABYV was by far the most prevalent virus, infecting about 83% of the samples analyzed (Table 2). The second most frequent virus was CYSDV, which infected about 31% of the melon samples. BPYV and WMV followed in relative importance, both with frequencies of around 20%. We found that MNSV, CMV, ZYMV, CVYV, and PRSV infected less than 6% of the total number of analyzed plants. These less prevalent viruses showed a sharp decrease between 2003 and 2004, falling from an infection rate of around 10% to less than 3% of the melon samples analyzed (Table 2). While CYSDV and WMV also decreased in frequency in 2004, CABYV registered a slight increase. BPYV also increased in prevalence in infected melon samples from around 15% in 2003 to 26% in 2004 (Table 2).

CABYV was also the most prevalent virus in squash, infecting more than 65% of the squash samples analyzed (Table 3). In squash, the next important viruses after

Table 1. Plasmids used for preparation of RNA probes

Virus ^a	Plasmid name	cDNA		Probe synthesis	
		Length	Genes	Restriction enzyme	Polymerase
CYSDV	pLM15	5 kbp	ORFs 1a and 1b	<i>Xho</i> I	Sp6
PRSV	pGEM-PRSV	1.2 kbp	NIa and NIb	<i>Sph</i> I	Sp6
BPYV	pBPYV	0.7 kbp	HSP70h	<i>Sac</i> I	T7
CABYV	pGEM-CABYV	0.6 kbp	CP	<i>Sph</i> I	Sp6
CMV	pRNA3-3a	0.5 kbp	ORF 3a	<i>Bam</i> HI	T7
CVYV	pLMAM48	1.5 kbp	NIb and CP	<i>Sph</i> I	Sp6
WMV	pQ2-1.6	1.6 kbp	NIb and CP	<i>Sal</i> I	T7
MNSV	pY2	4.3 kbp	Full length	<i>Sac</i> I	T7
ZYMV	pGEM-ZYMV	2.7 kbp	NIa, NIb and CP	<i>Pst</i> I	T7

^a *Beet pseudo-yellow virus* (BPYV), *Cucurbit aphid-borne yellows virus* (CABYV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Melon necrotic spot virus* (MNSV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV).

CABYV were CVYV and BPYV, infecting 28 and 21% of the samples, respectively. WMV, CYSDV, and ZYMV were detected in 3 to 6% of the samples, while CMV, MNSV, and PRSV were less prevalent, infecting less than 1.5% of the samples. In the 2004 growing season, many fewer squash plants than in 2003 were found to be infected by the viruses tested (Table 3), resulting in a higher variation in viral incidence in squash during these two consecutive years than in melon: CABYV fell from about 88 to 45% infection, while BPYV dropped even more sharply, from around 43% to less than 1% infection. WMV and CYSDV were not detected in 2004, although their incidence had been 13 and 9%, respectively, in 2003. Only the percentage of plants infected by CVYV (and to a lesser extent, PRSV and MNSV) increased in 2004 in comparison with 2003 (Table 3).

During the surveys, the proportion of plants showing yellowing symptoms was visually estimated for each plot. These observations, together with the results of the analysis of virus detection in samples,

provided an estimation of CABYV incidence in the plots considered. In 71% of the plots, the incidence of CABYV was greater than 20%; CABYV was absent in only one of the plots, while in another plot 100% of the plants showed symptoms of infection. Symptoms on melon plants infected by CABYV included yellowing and thickening of the basal and older leaves, which occasionally also showed slight epinasty, or resulted in flower abortion and a reduction in the number of fruits per plant (Fig. 1A to C). Frequently, the chlorotic basal leaves of older plants developed large interveinal necrotic areas. For squash, the symptoms included yellowing of the basal leaves and generalized chlorosis (Fig. 1D to F).

Multiple infections. The number of multiple infections was approximately 60% of the infected samples for both melon and squash. In melon, the most frequent double infection was CABYV/CYSDV, followed by CABYV/WMV and CABYV/BPYV. The last combination appeared only in 2004, possibly because of the higher incidence of BPYV (Table 4). In

2003, approximately 61% of infected squash samples were doubly infected, compared with about 26% in 2004 (Table 5). The most frequent double infection was CABYV/BPYV, followed by CABYV/CVYV. The CABYV/BPYV combination was not detected in 2004, most probably because of the drastic drop in BPYV. Similarly, the double infection by CABYV/CVYV increased at the same time as CVYV incidence increased. Among the triple infections, the most frequent association was BPYV/CABYV/CYSDV for both melon and squash (Tables 4 and 5).

RFLP analyses of CABYV isolates. To analyze the genetic variability of CABYV isolates in a fast and efficient way, RFLP analyses were performed. Thirty-four melon and squash samples that showed an intense hybridization signal for CABYV were selected for these studies. These samples included 21 melon (11 and 10 samples from 2003 and 2004, respectively) and 13 squash samples (7 and 6 samples from 2003 and 2004, respectively). cDNAs corresponding to the gene encoding the CP (approx. 600 bp) were prepared by RT-PCR from the 34 CABYV-infected samples and digested with 10 restriction enzymes. No restriction sites for *Dra*III, *Eco*RI, *Kpn*I, *Sac*I, *Sal*I, and *Xba*I were detected in the cDNAs. *Bam*HI cut only one of the 34 cDNA into two fragments. *Hind*III cut all cDNA samples analyzed into two fragments of approximately 0.2 and 0.4 kbp. On the other hand, digestion of samples with *Bcl*I resulted in two different patterns: Pattern 1 consisted of approximately 0.4 and 0.2 kbp fragments, while pattern 2 consisted of fragments approximately 0.4 and 0.1 kbp in size (Fig. 2A). From the total number of isolates studied, 28 corresponded to pattern 2 and six to pattern 1 (Table 6). The 0.1-kbp band consisted possibly of two bands of very similar size, although we cannot exclude that in these samples, additional *Bcl*I restriction sites exist that digest one of the two ≈0.1 kbp fragments into smaller DNA pieces not visible on agarose gels. Digestion of cDNA with the restriction enzyme *Fok*I also made it possible to classify the same samples into the same two groups: cDNA from six samples remained uncut (pattern 1), whereas cDNA from the other 28 samples were digested into two bands of ≈0.5 and ≈0.1 kbp (pattern 2) (Fig. 2B). There was a perfect correlation between patterns 1 and 2 found with restriction enzymes *Bcl*I and *Fok*I as illustrated in Figure 2 and detailed in Table 6. Nucleotide sequences of this DNA fragment for isolates M/2003/36.2 and M/2003/37.5 (18) also support these results (data not shown). Isolate Sq/2004/1.9, with a distinct *Bam*HI pattern, seemed to be a variant of pattern 2. No association could be identified between host (melon or squash) and type of RFLP pattern. Interestingly, during the 2003 epidemic episode, isolates

Table 2. Occurrence of viruses^a in melon grown in Murcia, Spain

Virus	Infected samples (% of infection) ^b		
	2003	2004	Total
CABYV	167 (78.4)	416 (85.4)	583 (83.3)
CYSDV	76 (35.7)	144 (29.6)	220 (31.4)
BPYV	31 (14.5)	125 (25.7)	156 (22.3)
WMV	61 (28.6)	88 (18.1)	149 (21.3)
MNSV	27 (12.7)	13 (2.7)	40 (5.7)
CMV	22 (10.3)	12 (2.5)	34 (4.9)
ZYMV	22 (10.3)	3 (0.6)	25 (3.6)
CVYV	21 (9.9)	0	21 (3.0)
PRSV	15 (7.0)	2 (0.4)	17 (2.4)

^a Viruses analyzed were: *Beet pseudo-yellows virus* (BPYV), *Cucurbit aphid-borne yellows virus* (CABYV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Melon necrotic spot virus* (MNSV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV). Viruses were diagnosed by molecular hybridization either in tissue prints or dot-blots as described in Marco et al. (25).

^b Total number of melon samples analyzed was 700: 213 during 2003 and 487 during 2004. Samples were from plants showing viruslike symptoms.

Table 3. Occurrence of viruses^a in squash grown in Murcia, Spain

Virus	Infected samples (% of infection) ^b		
	2003	2004	Total
CABYV	95 (88.0)	52 (44.8)	147 (65.6)
CVYV	25 (23.1)	38 (32.8)	63 (28.1)
BPYV	46 (42.6)	1 (0.9)	47 (21.0)
WMV	14 (13.0)	0	14 (6.2)
CYSDV	10 (9.3)	0	10 (4.5)
ZYMV	5 (4.6)	2 (1.7)	7 (3.1)
PRSV	0	3 (2.6)	3 (1.3)
CMV	1 (0.9)	0	1 (0.4)
MNSV	0	1 (0.9)	1 (0.4)

^a Viruses analyzed were: *Beet pseudo-yellows virus* (BPYV), *Cucurbit aphid-borne yellows virus* (CABYV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Melon necrotic spot virus* (MNSV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV). Viruses were diagnosed by molecular hybridization either in tissue prints or dot-blots as described in Marco et al. (25).

^b The total number of squash samples analyzed was 224: 108 during 2003 and 116 during 2004. Samples were from plants showing viruslike symptoms.

with RFLP patterns 1 and 2 were found, while in 2004 only isolates with RFLP pattern 2 were found (Table 6).

DISCUSSION

In this paper, we have analyzed a large number of melon and squash samples to determine the presence of nine viruses belonging to six different genera. Hybridization of dot-blot and tissue prints of petiole cross-sections were used primarily because of the availability in our labora-

tory of most of the probes utilized in this study. We have not done a systematic comparison of the detection methods used here with others more commonly used such as double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) and RT-PCR, but we have found some advantages: hybridization in tissue prints does not require preparation of the sample prior to membrane printing, thus saving time and reducing costs. In our experience, this technique is particularly

well suited and very sensitive for detection of phloem-limited, yellowing-inducing viruses such as BPYV, CABYV and CYSDV, but it is less sensitive than dot-blot for detection of the other viruses (data not shown). Therefore, each sample was analyzed by dot-blot hybridization using each of the probes described above, and samples from plants showing yellowing symptoms were also analyzed by tissue-printing with probes for BPYV, CABYV and CYSDV. The data presented

Fig. 1. Symptoms in plants infected with *Cucurbit aphid-borne yellows virus* (CABYV). In melon, yellowing and thickening of the basal leaves (A) and necrosis of basal leaves of older plants (B) was frequent. Melon (C) and squash (D) crops of southeastern Spain often showed CABYV incidences close to 100%. Symptoms in squash included interveinal chlorosis (E) and intense yellowing of the basal leaves and reduced plant growth (F) compared with healthy plants (on the right).

here show that CABYV was the most prevalent and widespread virus in open field crops of the southeastern part of Spain during 2003 to 2004. CABYV was

first described by H. Lecoq and co-workers in 1992 in France, where it affected open-field cucurbit crops (20). CABYV was later detected in Italy, Greece, Tunisia,

Algeria, Lebanon, Turkey, Sudan, Nepal, Taiwan, China, Reunion Island, Swaziland, Brazil, Honduras, Spain, and California (U.S.A) (2,18–22,28). Extensive surveys conducted by Lecoq and co-workers have shown that CABYV is one of the most common cucurbit viruses in open-field crops in a great diversity of areas and environments (19,21).

Due to the similarity of symptoms induced by CABYV with those induced by other viruses and factors including abiotic stress, we suppose that in Spain the presence of CABYV has remained unnoticed during some years. This could also be the case in other Mediterranean countries (19). In agreement with our findings, field-grown cucurbit crops (cucumber, melon, squash, and watermelon) from Lebanon also showed high CABYV incidence and a similarly high incidence of ZYMV (1). Infections with WMV, PRSV, CMV, and MNSV were also detected, but at much lower level. In our survey, the second most important virus in melon was CYSDV, followed by BPYV. High incidences of CYSDV in melon and cucumber greenhouses have been previously reported in the southeastern coast of Spain (5,30). In our survey, WMV occurred in melon as frequently as BPYV, followed in importance by MNSV and CMV, both of similar incidence, and by ZYMV and CVYV. The least prevalent virus was PRSV. A similar result with WMV and CMV was obtained in a survey of melon fields for mosaic-inducing viruses in 1995 to 1996 in the region of Murcia, when the WMV infections were detected twice as often as CMV infections (24). In this and other studies in some other important melon-producing regions of Spain, a higher incidence of

Table 4. Multiple infections in melon

Infection ^b	Infected samples (% of total of infected samples) ^a		
	2003	2004	Total
Double	90 (44.6)	209 (47.1)	299 (46.3)
CABYV-CYSDV	48 (23.8)	69 (15.5)	117 (18.1)
CABYV-WMV	29 (14.4)	69 (15.5)	98 (15.2)
CABYV-BPYV	-	52 (11.7)	52 (8.0)
Triple	33 (16.3)	72 (16.2)	105 (16.3)
BPYV-CABYV-CYSDV	29 (14.4)	69 (15.5)	99 (15.3)
More than three	21 (10.4)	1 (0.2)	22 (3.4)
PRSV-ZYMV-MNSV-WMV-CABYV	1 (0.5)	1 (0.2)	2 (0.3)
PRSV-ZYMV-MNSV-WMV-CABYV-CVYV	4 (2.0)	-	4 (0.6)
PRSV-ZYMV-MNSV-CMV-WMV-CABYV-CVYV	2 (1.0)	-	2 (0.3)
Total	144 (71.3)	282 (63.5)	426 (65.9)

^a The total number of melon samples infected by at least one of the viruses considered was 202 during 2003 and 444 during 2004.

^b *Beet pseudo-yellow virus* (BPYV), *Cucurbit aphid-borne yellows virus* (CABYV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Melon necrotic spot virus* (MNSV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV). Only the most frequent combinations are indicated.

Table 5. Multiple infections in squash

Infection ^b	Plants infected (% of total infected samples) ^a		
	2003	2004	Total
Double	63 (60.6)	19 (26.4)	82 (46.6)
CABYV-BPYV	42 (40.4)	-	42 (23.9)
CABYV-CVYV	11 (10.6)	18 (25.0)	29 (16.5)
Triple	14 (13.5)	3 (4.2)	17 (9.7)
BPYV-CABYV-CYSDV	4 (3.8)	-	4 (2.3)
Total	77 (74.0)	22 (30.6)	99 (56.2)

^a The total number of melon samples infected by at least one of the viruses considered was 104 during 2003 and 72 during 2004.

^b *Beet pseudo-yellow virus* (BPYV), *Cucurbit aphid-borne yellows virus* (CABYV), *Cucumber vein yellowing virus* (CVYV), and *Cucurbit yellow stunting disorder virus* (CYSDV). Only the most frequent combinations are indicated.

Fig. 2. Restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified coat protein gene of *Cucurbit aphid-borne yellows virus* (CABYV) isolates. **A**, Ethidium bromide-stained agarose gel showing restriction analysis with *Bcl*I of seven different samples. Two different restriction patterns can be observed with bands of ≈ 0.4 and ≈ 0.1 kb, and of ≈ 0.4 and ≈ 0.2 kb. **B**, Digestion of the same seven samples with *Fok*I. Four samples have been digested into fragments of ≈ 0.5 and ≈ 0.1 kb, while the remaining three samples are not cut by this enzyme. On the right, the size in base pairs (bp) of a DNA ladder is indicated. The samples are designated M (for melon) or Sq (for squash), the year of isolation, and the field and sample numbers.

CMV than of WMV was found (3,24). Also, in Turkey, another Mediterranean country important for its open-field cucurbit crops, WMV was shown to be the most important virus in melon and squash, followed in both crops by CMV and ZYMV (33). We must point out that in our survey the second most important virus in squash was CVYV. This virus has recently been reported in greenhouse-grown cucumber in Almería (southeastern Spain), where its occurrence caused great alarm among cucumber growers (6). CVYV has extended to other crops such as melon, squash, and watermelon in this and other Spanish regions, as well as in southern Portugal, where it is threatening cucurbit production (23,26). Other European countries are taking measures to prevent the spread of this virus (7,23).

For melon and squash, we detected multiple infections in approximately 60% of the infected samples. The most common double infection in melon was CABYV/CYSDV, closely followed by CABYV/WMV. In squash, the most common double infections were CABYV/BPYV and CABYV/CVYV. Earlier surveys have pointed to lower percentages of multiple infections. In 1998 in Lebanon, approximately 13% of melon and 30% of squash plants showed multiple infections, with ZYMV/CABYV being almost the

only virus combination found (1). In other studies in which CABYV was not included, double infections ranged between 0 and 15%: These studies included viruses such as ZYMV, WMV, CMV, and PRSV, and cucumber, melon, and watermelon as hosts. The highest value (15%) was found for cucurbits of Turkey doubly infected with ZYMV and WMV (24,33). Enhanced symptom expression has been associated with mixed infections. This appears to be due to synergistic effects occurring for a number of virus combinations and is probably related to the silencing suppression ability of the viruses involved (16). For viruses in the family *Luteoviridae*, synergistic effects have been described (32). The high incidence of CABYV observed here, together with the high number of double and multiple infections (especially the double infections with the two emergent viruses CABYV-CVYV), suggests that CABYV may become an important threat for cucurbit crops in the future.

RFLP analysis with *BclI* and *FokI* suggested that the CABYV isolates analyzed can be divided into two genetic groups with six isolates that belong to group 1 (pattern 1) and 28 isolates that belong to group 2 (pattern 2). Interestingly, both genetic types were present during the 2003 epidemic, but in 2004 only isolates of

group 2 were found, suggesting that group 2 had displaced group 1. This is an attractive hypothesis, but clearly surveys lasting only 2 years are not sufficient to sustain such a hypothesis and further studies are required. On the other hand, during 2003, grouping did not correlate with the original host or with the place of isolation, suggesting that CABYV does not have a host preference. Differences in the ability to colonize hosts alternative to melon and squash, and/or differences in transmission efficiencies by different aphids, may explain geographical and temporal patterns of strain distribution (14), but again these aspects remain to be studied.

In France, reductions of about 50% in cucumber production and 40% in melon production were observed due to the abortion of flowers and the negative effects on fruit quality as the result of infection by CABYV (20). In some highly infected fields, we also observed loss of fruit set due to flower abortion that resulted in a reduction in the final fruit yield (data not shown). Therefore, the economic impact of infection with CABYV may be very high. Currently, control of aphid vectors is the strategy used to prevent infection with CABYV. This classical method has negative effects, such as the increasing risk of selection of resistant aphid populations and the potential damage to the environment. The use of physical barriers such as plastic covers for the control of aphids during the early stages of plant development results in a delay of a few weeks in the establishment of infections but does not confer efficient protection (19). The use of these covers is frequent in early plantations in the region studied here. In our experience, the use of such protection confers a good level of control for nonpersistently transmitted viruses (e.g., CMV, ZYMV) but not for persistently transmitted viruses such as CABYV. An important option for disease control would be the use of cultivars that carry genetic resistance to the virus. Several resistance sources for CABYV in melon have been described (10,11). However, to our knowledge, melon cultivars with resistance to CABYV have not been produced on a commercial basis.

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Table 6. Restriction fragment length polymorphism (RFLP) patterns found for the different isolates studied

Isolate	Host	Year of isolation	Restriction enzyme			Group
			<i>BclI</i>	<i>FokI</i>	<i>BamHI</i>	
M/2003/11.5	Melon	2003	0.4 + 0.2	0.6	0.6	1
M/2003/12.7	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2003/13.7	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2003/24.2	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2003/24.6	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2003/36.2	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2003/36.8	Melon	2003	0.4 + 0.2	0.6	0.6	1
M/2003/37.2	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2003/37.5	Melon	2003	0.4 + 0.2	0.6	0.6	1
M/2003/39.5	Melon	2003	0.4 + 0.2	0.6	0.6	1
M/2003/39.9	Melon	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2003/5	Squash	2003	0.4 + 0.2	0.6	0.6	1
Sq/2003/3.1	Squash	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2003/3.3	Squash	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2003/4.6	Squash	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2003/7.2	Squash	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2003/8.1	Squash	2003	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2003/8.3	Squash	2003	0.4 + 0.2	0.6	0.6	1
M/2004/7.3	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/8.5	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/19.17	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/22.11	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/24.4	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/25.2	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/28.11	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/30.9	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/31.9	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
M/2004/39.14	Melon	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2004/1.9	Squash	2004	0.4 + 0.1	0.5 + 0.1	0.4 + 0.2	2
Sq/2004/2.3	Squash	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2004/2.6	Squash	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2004/5.5	Squash	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2004/5.8	Squash	2004	0.4 + 0.1	0.5 + 0.1	0.6	2
Sq/2004/9.8	Squash	2004	0.4 + 0.1	0.5 + 0.1	0.6	2

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