European Journal of Plant Pathology **105:** 211–215, 1999.

Short communication

Occurrence of cucurbit yellow stunting disorder virus (CYSDV) and beet pseudo-yellows virus in cucurbit crops in Spain and transmission of CYSDV by two biotypes of *Bemisia tabaci*

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Accepted 28 December 1998

Key words: closterovirus, crinivirus, *Trialeurodes*, whiteflies

Abstract

The relative occurrence in Spain of two whitefly transmitted closteroviruses causing similar yellowing diseases in melon and cucumber greenhouse crops was studied. Based on a RT-PCR assay, a 1994–1997 survey of Spanish greenhouses showed that the recently described *Bemisia tabaci*-transmitted cucurbit yellow stunting disorder virus (CYSDV) has displaced the *Trialeurodes vaporariorum*-transmitted beet pseudo-yellows virus (BPYV), a virus that was present in the area since the late 1970s. The CYSDV transmission rates by each of the two biotypes of *B. tabaci* present in Spain were compared. The results showed that the ubiquitous 'B' biotype and the resident 'Q' biotype (found in Spain and Portugal) were able to transmit CYSDV with similar efficiency.

The out-of-season cultivation of cucurbits in plastic greenhouses has major economic importance in many Mediterranean countries. Only in Spain the cultivation of melon (*Cucumis melo* L.) and cucumber (*Cucumis sativus* L.) amounts for 16,000 ha of plastic greenhouses along the southeastern (SE) coast. Since the late 1970s these two crops have been seriously affected by yellowing diseases transmitted by whiteflies. Two viruses have been associated with these diseases in Spain. First, a virus transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum* Westwood) was reported to occur in greenhouse-grown cucurbits in several countries including Spain (Jordá-Gutiérrez et al., 1993; Lot et al., 1982, Soria et al., 1991; van Dorst et al., 1980, Yamashita et al., 1979). The virus shared biological properties with the closterovirus beet pseudo-yellows virus (BPYV) (Duffus, 1965; Liu and Duffus, 1990). Indeed, partial sequences of the HSP70-homolog gene of several isolates of this closterovirus, including one isolate from Spain and the prototype BPYV isolate, were virtually identical (Coffin and Coutts, 1995; Livieratos et al., 1998; Tian et al., 1996). This is also the case for a *T. vaporariorum*-transmitted closterovirus obtained from cucumber in France (termed cucumber chlorotic spot virus, CCSV) (Woudt et al., 1993), that has been found to differ only by 1% from the sequence reported for BPYV in the region mentioned above (B. Woudt, unpublished data), indicating that CCSV should also be considered an isolate of BPYV.

In addition to BPYV, since the early 1990s a second closterovirus, cucurbit yellow stunting disorder virus (CYSDV), has been associated with these diseases in Spain. CYSDV is a bipartite closterovirus transmitted by the tobacco whitefly *Bemisia tabaci* Genn. (Célix et al., 1996) and its appearance in the area coincided with the displacement of *T. vaporariorum* as the prevalent whitefly species in the greenhouses

of SE Spain. The relative occurrence of BPYV and CYSDV in greenhouses affected by the yellowing disease is unknown due to the lack of efficient diagnostic tools. This is an important issue since the genetic resistance found for CYSDV in melon germplasm is virus-specific and does not hold against Spanish isolates of BPYV (M.L. Gómez-Guillamón, pers. comm.). Recently, oligonucleotide primers able to differentiate between CYSDV and BPYV in RT-PCR assays have been described (Célix et al., 1996; Livieratos et al., 1998).

A recent study has shown that Spanish populations of *B. tabaci* are composed of two genetic types (Guirao et al., 1997). One corresponds to populations of the ubiquitous 'B' biotype (also known as *B. argentifolii*) introduced in several countries in the past few years (Perring et al., 1993). The other biotype has been termed 'Q' biotype and seems specific of Spain and Portugal (Guirao et al., 1997). Whether both biotypes found in Spain have the ability to transmit CYSDV is unknown but data on other *B. tabaci*-transmitted closteroviruses suggest that transmission efficiency can vary widely according to the biotype (Wisler et al., 1998).

During 1994–1997 we sampled cucumber and melon plants with yellowing symptoms from Spanish greenhouses, divided into four geographical areas (Table 1): the main cultivation area near Almería (divided into two regions, East and West) and two additional cultivation areas more than 100 km apart from Almería greenhouses (Málaga and Murcia). Samples were taken in the winter (mostly cucumber crops) and in the spring (mostly melon crops). A leaf piece of about 0.2 g was used for preparation of total RNA (Célix et al., 1996). CYSDV was detected by RT-PCR using previously described oligonucleotide primers 410L and 410U (Célix et al., 1996). The expected PCR product was a 0.48 kb fragment corresponding to the HSP70-homolog gene of CYSDV. BPYV was detected with oligonucleotide primers 175 (5′CTTAGTGATTCCAAGCC 3′) and 172 (5′CTTTGTGCTCCCAACTCAAC 3′). These primers were designed from the sequence of the BPYV isolate formerly known as CCSV (Woudt et al., 1993). Primer 172 was used in the reverse transcription reaction of total RNA extracts, followed by PCR amplification with both 172 and 175 oligonucleotides with the reaction conditions described by Célix et al. (1996). The expected PCR product for oligonucleotides 175 and 172 was a 0.78 kb fragment from the open reading frame 1 b of the BPYV genome (Woudt et al., 1993).

Table 1. Occurrence of CYSDV in greenhouse cucumber (*Cucumis sativus*) and melon (*Cucumis melo*) crops in Spain[a]

Sampling date	Area	Cucumber	Melon
Spring 1994	Almería W		2/6
	Almería E		0/2
Winter 1994	Almería W	22/31	7/10
	Almería E	4/7	0/5
	Málaga	5/5	2/7
Spring 1995	Almería W	3/6	1/10
	Almería E		10/14
Winter 1995	Almería W	11/12	
	Almería E	3/4	
	Málaga	3/4	
	Murcia	2[b]/2	
Spring 1996	Almería W	2/3	0/24
	Almería E		0/4
Winter 1996	Almería W	16/21	
	Almería E	7/8	
	Málaga	0/1	
Spring 1997	Almería W	5/5	2/7
Winter 1997	Almería W	4/6	3/3
	Almería E	6[c]/9	
	Málaga	3/3	2/2
	Murcia	5/7	0/2
	Total	101/134	29/96

[a] Data are given as number of positive samples in RT-PCR assays out of number of tested samples.
[b] Both samples tested also positive for BPYV.
[c] One of these samples tested also positive for BPYV.

The specificity of the RT-PCR detection assay is illustrated in Figure 1. Each pair of oligonucleotides selected amplified only the homologous virus and did not react with non-infected extracts. The oligonucleotides used for detecting BPYV successfully amplified the expected PCR fragment from two isolates of this virus (obtained from cucumber plants in Spain and France) maintained in the laboratory. We also tested the simultaneous use of both pairs of oligonucleotides in the RT-PCR reactions and the specificity of the assay was maintained (not shown). Also, this modification of the RT-PCR assay allowed us to detect mixtures of nucleic acid extracts from CYSDV and BPYV infected controls in a single RT-PCR reaction (not shown).

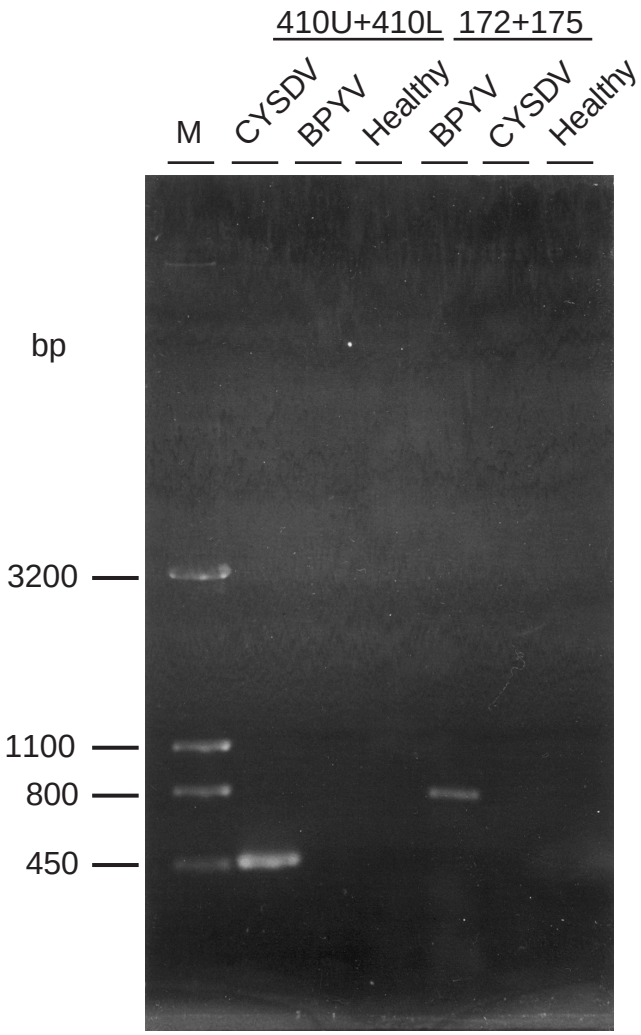

Figure 1. Assay for specific detection of whitefly-transmitted closteroviruses causing yellowing disease of cucurbits in Spain. Agarose gel of RT-PCR products from total RNA extracts prepared from cucumber plants artificially inoculated with CYSDV (lanes CYSDV) or with BPYV (lanes BPYV) and from uninoculated plants (lanes Healthy). RT-PCR was performed with CYSDV-specific oligonucleotide primers 410L and 410U that amplify a PCR product of 0.48 kb from CYSDV RNA 2 or with primers 172 and 175 that amplify a 0.78 kb fragment from the open reading frame 1 b of BPYV.

A total of 96 melon samples were analyzed and none was found to be infected with the *T. vaporariorum*-transmitted virus BPYV (not shown). In contrast, near 30% of the samples were infected by the *B. tabaci*-transmitted virus CYSDV (Table 1). The large proportion of yellowing plants of melon that tested negative with both oligonucleotide pairs prompted us to perform the diagnostic assay with an additional pair of CYSDV-specific primers, based in a region of the CYSDV genome corresponding to the closterovirus p59 gene (our unpublished results). This assay gave identical results (not shown) to those obtained with CYSDV primers 410U and 410L, summarized in Table 1. The possibility that other viruses causing yellowing of cucurbits were present in melon crops was also tested. Lettuce infectious yellows virus (LIYV) is a *B. tabaci*-transmitted closterovirus reported only in North America, causing yellowing in several crops including melon (Duffus et al., 1986; Wisler et al., 1998). A luteovirus, cucurbit aphid-borne yellowing virus (CABYV), causes yellowing symptoms in field-grown melon crops in Europe (Lecoq et al., 1992). The

presence of LIYV or CABYV in field samples was tested by Western blot analysis of total protein extracts (Rodríguez-Cerezo and Shaw, 1991) with anti-LIYV or anti-CABYV polyclonal sera (the gift of B. Falk, University of California, Davis) diluted 500-fold. All melon samples were tested and were negative for LIYV by Western blot. The presence of CABYV was occasionally tested and never found in the greenhouses. This was expected since aphids and aphid-borne viruses rarely occur in the plastic-protected crops of the area.

In samples from cucumber plants, only 3 out of 134 tested positive for BPYV and in all cases corresponded to plants doubly infected with BPYV and CYSDV (Table 1). In contrast, about 75% of the cucumber samples were positive for CYSDV, a much larger proportion than in the case of melon plants. LIYV was not detected by Western blot analysis in cucumber plants. As a control for all experiments, leaves were often collected from symptomless plants in melon and cucumber greenhouses and never found to be infected by either of the closteroviruses tested by RT-PCR. Finally, natural infection of zucchini squash by CYSDV was confirmed by RT-PCR in a single greenhouse in the area of East Almería (not shown).

The two biotypes of *B. tabaci* present in Spain ('B' and 'Q') were tested for CYSDV transmission. A first experiment showed that both biotypes were able to transmit CYSDV, and subsequent experiments were designed to compare the efficiency of transmission. The results (Table 2) showed a similar efficiency for both biotypes in transmitting CYSDV under controlled conditions. The probability of a single whitefly transmitting CYSDV can be estimated from these data (Swallow, 1985) and gives values of 0.045 for the 'B' biotype and 0.055 for the 'Q' biotype.

Our data show that BPYV, present in the Spanish greenhouse area since the early 1980s, has been practically displaced by CYSDV. This situation coincides with the prevalence of *B. tabaci* in greenhouses over *T. vaporariorum*. Whether or not CYSDV infection may cross-protect from BPYV infection and vice versa is not known, but the finding of samples with mixed infections argues against this possibility. CYSDV was probably introduced in the area of Almería in the late 1980s and has spread throughout the melon and cucumber greenhouse area. Only a few BPYV-infected cucumber samples were identified (Table 1) and some originated from greenhouses of Murcia, a region isolated from the main greenhouse area of Almería.

Table 2. Efficiency of transmission of CYSDV by two biotypes of *Bemisia tabaci*[a]

Transmission rate (%)[b]	Insects per plant			
	60	30	15	5
'Q' biotype	100	85.7	68	13.3
'B' biotype	100	100	70	5.5

[a] Both biotypes were kept in cucumber plants for several generations previous to the transmission experiments. Acquisition and inoculation access times were fixed (48 and 72 h respectively).
[b] Data are average of 2 experiments.

Although we cannot rule out the presence of unknown viruses, our results on the occurrence of BPYV and CYSDV suggest that in many cases the yellowing symptoms of greenhouse melon crops in Spain may not be due to virus infection. The correlation between symptoms and CYSDV infection in cucumber was much higher suggesting that nutrient deficiencies or other physiological factors are less frequently observed in cucumber than in melon. Our assay was also able to detect CYSDV and not BPYV in cucumber samples from Israel and Jordan (not shown) indicating that CYSDV occurs in several Mediterranean countries. Thus it appears that CYSDV is an emerging and important pathogen for cultivated cucurbits in this area. It is remarkable that zucchini squash and watermelon greenhouse crops in Spain are not widely affected by the yellowing disease, although they are experimental hosts for both CYSDV and BPYV. It could be due to tolerance of the cultivated varieties or to slow symptom development in field conditions. In addition, epidemiological factors such as the time of planting may be important for virus incidence. In any case we have been able to detect CYSDV by RT-PCR in one commercial zucchini greenhouse in Almería, adding to the importance of CYSDV as a pathogen in the area. This is the first report of CYSDV naturally infecting zucchini squash crops.

Our results are important for the management of the yellowing disease of cucurbits in Spain. Chemical control of the vectors has not been effective to prevent the spread of these closteroviruses. Genetic resistance is sought in melon and cucumber germplasm as a means of controlling these diseases. Monogenic, dominant resistance against CYSDV has been recently described in melon germplasm but has been proven uneffective against BPYV (M.L. Gómez-Guillamón, pers. comm.) and this is an important finding since

BPYV and its vector have not completely disappeared from the area. Conversely, BPYV-tolerant melon lines have been described but whether the tolerance is maintained against CYSDV infection has not been tested (Esteva et al., 1988). In any case, a genetic approach for controlling both viral diseases will have to take into account the relative incidence of each closterovirus in a particular region.

We have shown that under controlled conditions CYSDV is transmitted with similar efficiency by the two biotypes of *B. tabaci* that have been described in Spain, the ubiquitous 'B' biotype and the resident 'Q' biotype (Guirao et al., 1997). In contrast, the American resident biotype 'A' is an inefficient vector of CYSDV when compared with the 'B' biotype (Wisler et al., 1998). In other whitefly-transmitted closteroviruses, different situations have been described ranging from highly biotype-specific transmission to transmission by several whitefly species (Wisler et al., 1998). The variability in the specificity of the closterovirus-vector association raises interesting questions on the mechanisms of closterovirus transmission by whiteflies and could be an important factor to understand the different geographical distribution of these viruses.

Acknowledgements

This work was funded by Grant 95-011-0P from Spanish CICYT and by a grant from Novartis Seeds.

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