Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2023, Vol. 7 (1), 74-84
https://doi.org/10.29329/ijiaar.2023.536.5


Original article

# Research on Drawing Attention to Overlooked Viruses in Plant Viruses Causing Yellowing-Type Symptoms in *Cucurbitaceae*

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**Abstract**

A single-stranded, positive-sense plant RNA virus called the Cucurbit chlorotic yellows virus (CCYV, *Crinivirus, Closteroviridae*) is comprised of RNA1 and RNA2. It is semipersistently transmitted by the whitefly *Bemisia tabaci* biotypes MEAM1 and MED. In 2004, CCYV was discovered on melon plants in Japan. Several other cucurbit species, as well as a range of non-species, were subsequently reported from countries such as Saudi Arabia, California, Israel, Taiwan, Sudan, Lebanon, Iran, Greece, Türkiye, Egypt, Spain, and China. Whitefly populations are frequently encountered in cucurbit cultivating areas. Virus plant diseases attributed to whiteflies are common in squash cultivation in the open field and greenhouses in Türkiye, especially in cucumbers and squash. *Cucumber vein yellowing virus* (CVYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Tomato leaf curl New Delhi virus* (ToLCNDV), *Squash vein-yellowing virus* (SqVYV) are the leading virus diseases transmitted by *Bemisia tabaci*. Symptoms caused specifically by these viral diseases are of the yellowing type, although they differ as intervascular yellowing, yellowing of old or young leaves, and upward curling. Besides these viruses, CCYV, a problem in cucurbits, is carried by whitefly like others, causes yellowing type symptoms, and its first record in cucumber was reported in 2017 in Türkiye. However, this virus is ignored in the production of cucurbits, it is a problem in production areas. There are major viruses cause similar symptoms with the presence of the Cucumber chlorotic yellows virus is overlooked in the symptomatological observation. In this study, we tried to reveal the difference of CCYV, which is encountered in cucurbit growing areas in Antalya and whose presence was detected by RT-PCR, from other viruses and emphasized its importance as a virus that should be considered in classical breeding studies.

**Keywords:** CCYV, Cucurbits, Plant Viruses, Yellowing-Type Symptoms.

**Received:** 19 September 2022 * **Accepted:** 13 March 2023 * **DOI:** https://doi.org/10.29329/ijiaar.2023.536.5

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## INTRODUCTION

The *Cucurbitaceae* family contains over 800 plant species in 120 genera (Welbaum, 2015), which are herbaceous plants grow as annuals or perennials in temperate and tropical climates. Cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thunb.), pumpkin (*Cucurbita maxima* Duch. and *Cucurbita moschata* Duch.), and zucchini (*Cucurbita pepo* L.) are the most cultivated cucurbit crops in Mediterranean region. Cucurbit-producing countries should improve the quality and quantity of their production due to the economic importance of these crops (Radouane et al. 2021).

Cucurbits are vulnerable to a wide range of pests and pathogens including viruses. In the Mediterranean region, approximately 28 plant viruses are currently threatening cucurbit crop production (Lecoq and Desbiez, 2012).

Virus diseases are a worldwide issue for *Cucurbitaceae* and a major limitation to cucurbit production. Cucurbit viruses can infect a wide range of plants from various genera and families, with only a few viruses being specific to cucurbit species. Cucurbit viruses reduce plant vitality, flowering, and fruit production (Fidan et al. 2012).

The emergence of new virus diseases has become more common as pathogens evolve and their genetic diversity grows. Vectors play important roles in virus evolution through pathogen dispersion in a variety of plants and zones, and ecosystem simplification, expanding trade, and movement are also important factors in virus dispersion (Radouane et al. 2021).

In the proceeding of coevolution, plant viruses have established particular interactions with insect vectors. Nearly 80% of the plant viruses depend on vectors for transmission. Infection by a virus may alter the phenotypic and volatiles of the host plants as well as the insect vectors' behaviors (He et al. 2021).

Viruses are responsible for the majority of emerging plant diseases. This phenomenon is most likely caused by two factors: the plasticity of virus genomes and their efficient dissemination, the latter of which frequently relies on vector organisms. The situation is compounded by the limited set of controls available for virus-induced diseases. Intensive vegetable production is distinguished by a rapid turnover of cultivars and cultural practices, as well as by global seed and product trade. Because of the changing environment, new viruses and vectors may emerge (Navas-Castillo et al. 2014).

The efficient transmission of viruses has a significant impact role on the impact and outcome of epidemics. The presence and/or expansion of vector geographical and host ranges are the main risk determinants in this regard, and our ability to interfere with vector-mediated virus dissemination is still quite limited (Navas-Castillo et al. 2014).

One of the most significant agricultural pests and the most effective plant virus vectors in the world is *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (De Barro et al. 2011). According to earlier research (Jones 2003, Bragard et al. 2013, Polston et al. 2014), *B. tabaci* transmits more than 500 species of plant viruses from five families and five genera. Some of these viruses have seriously harmed agricultural production and resulted in financial losses (He et al. 2021).

Cucurbit production in Western Mediterranean countries, for example, has been severely harmed by a succession of epidemics caused by Cucurbit yellow stunting disorder virus (CYSDV; Célix et al., 1996), Cucumber vein yellowing virus (CVYV; Cuadrado et al., 2001), and Cucurbit aphid-borne yellows virus (Kassem et al., 2013).

Cucurbit chlorotic yellows virus (CCYV *Crinivirus*, *Closteroviridae*) is a single-stranded, positive-sense plant RNA virus that comprised of RNA1 and RNA2. Replication-related proteins with papain-like protease, methyltransferase, RNA helicase 1, and RNA-dependent RNA polymerase (RdRp) motifs are encrypted by RNA1. A homolog of the cellular 70-kDa heat-shock protein (HSP70h), the 59-kDa protein, the coat protein (CP), minor coat protein (CPm), and various other small proteins are all encoded by RNA2. The molecular criteria for species demarcation are determined by comparing the amino acid sequences of CP, CPm, and HSP70h. Species are determined to be distinct if there is a difference of greater than 10%. Two C*riniviruses* that infect cucurbits that are currently spread by whiteflies are BPYV and CYSDV (Okuda et al. 2010). CCYV has been reported from many different countries in cucurbit growing and whitefly populations. Reported by Abrahamian et al. from Lebanon. (2012), Al-Saleh et al. (2015) from Saudi Arabia, Amer et al. from Egypt (2015), Orfanidou et al. from Turkiye (2017), Salem et al. from Jordan (2020), Kwak et al. from Spain (2021) on cucumber plants. Also, reports were recorded by Zeng et al. from China (2011), Wintermantel et al. from NewWorld (2019), Cho et al. from Korea (2021), Hernandez et al. from Texas (2021) on melons, as well as Kumar et al. from India (2021) for pumpkins and Jailani et al. from USA (2021) on watermelon. Apart from single infections, many different mixed infection records were found. CCYV infections reports such as; on Cucurbits in Taiwan (Huang et al. 2010), on cucumber, melon, and watermelon in China (Gu et al.2011), on Muskmelon and Cucumber in Sudan (Hamed et al. 2011), on Cucumber, Melon, and Squash in Iran (Bananej et al. 2013), on Cucumber, Melon, and Watermelon in Greece (Orfanidou 2017), on cucumber and zucchini in Algeria (Kheireddine et al. 2020), Watermelon and Zucchini in the Canary Islands, Spain (Alfaro-Fernández et al. 2022) are recorded. CCYV infection records have been reported in some weeds as well as crop plants like wild radish (*Raphanus raphanistrum* L.) in USA (Kavalappara et al. 2022). According to the results of the research in the literature review, reports that CCYV and CYSDV infections were recorded as a mix in melon (Mondal et al. 2021) and other cucurbits (Mondal et al. 2022) were also recorded.

## MATERIAL AND METHODS

### Material

#### *Sample collection*

Viral disease agents encountered in field observations and cucurbit growing areas were investigated. In the fields and greenhouses where cucurbits are grown, plants showing yellowing type were selected and samples were taken from different plants. Especially, samples were taken from areas where whiteflies have the potential to carry cucurbit viruses effectively.

### Methods

#### *Total RNA extraction and RT-PCR:*

For virus disease detection, total RNA was extracted from collected zucchini leaf sample using the CTAB extraction method. PCR and RT-PCR studies were carried out based on those nucleic acids and using specific coat protein primers to possible cucurbit viruses (Table 1).

**Table 1.** Virus diseases tested in PCR studies, primer sequences, molecular size of the amplicons and annealing temperatures.

| *Virus species* | *Primer sequences* | *Amplicon size (bp)* | *Annealing (°C)* | *Reference* |
|---|---|---|---|---|
| Cucurbits yellow stunting disorder virus | CYSDV-F: AGTGACATGCCTAACTGTTACTT<br>CYSDV-R: ATAGCTGCTGCAGATGGTTC | 364 | 54 | (Fidan et. al., 2012) |
| Cucumber vein yellowing virus | CVYV-F: AGCTAGCGCGTATGGGGTGAC<br>CVYV-R: GCGCCGCAAGTGCAAATAAAT | 450 | 55 | (Fidan et. al., 2012) |
| Beet pseudo yellows virus | BPYV-F: TCGAAAGTCCAACAAGACGT<br>BPYV-R: CTGATGGTGCGCGAGTG | 251 | 54 | (Fidan et. al., 2012) |
| Tomato leaf curl New Delhi virus | To-B1-F: GAAACACAAGAGGGCTCGGA<br>To-B1-R: GCTCCACTATCAAAGGGCGT | 677 | 55 | (Sáez et. al., 2016) |
| Cucurbit chlorotic yellows virus | CCYV-CPF: TCCCGGTGCCAACTGAGACA<br>CCYV-CPR: TACGCGCGGCAGAGGAATTT | 375 | 55 | (Kavalappara et. al 2021) |

The targeted regions were amplified using Xpert One-Step RT-PCR Kit and 2X Mastermix PCR kit (Grisp, Portugal) in a thermal cycler (Biorad, ABD) according to manufacturer's protocol. For RT-PCR analyses. The temperature conditions for amplification reactions were as follows: for cDNA

synthesis by 1 cycle for 15 min at 45°C and 1 min at 95°C; by 35 cycles of 95°C for 10 s, 54°C (CYSDV and BPYV), 55°C for (CVYV and CCYV) 10 s and 72°C for 15 seconds. The final extension was at 72°C for 5 min. Due to ToLCNDV is a DNA based plant virus, PCR method carried out according to DNA amplification protocol: initial denaturation for 3 min at 94°C, followed by 35 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 15 seconds. Amplified products were visualized and photographed using DNA documentation gel analysis (Biorad, ABD) by agarose gel electrophoresis and stained with DNA stain dye (50 µg/ml). DNA Ladder 100 bp (Grisp, Portugal) was used to determine the size of PCR amplified cDNA products.

## RESULTS

### Symptomatologic results

13 individual leaf samples from plants in a commercial field in Antalya that had yellowing, interveinal chlorosis, and mottling were collected throughout sampling sessions in June 2022. The observed symptoms, such as chlorotic yellowing and vein clearing, resembled those caused by whitefly-transmitted viruses such as the *Ipomovirus* (*Potyviridae*) *Cucumber vein yellowing virus* (CVYV), *Begomovirus* (*Geminiviridae*) Tomato leaf curl New Delhi virus (ToLCNDV), Cucurbit yellow stunting disorder virus (CYSDV), Beet pseudo-yellows virus, (BPYV) and the Criniviruses (Closteroviridae) (CCYV). Our symptomatogenic results that, cucurbits demonstrate brittleness, thickness, and interveinal yellowing of the leaves, which are similar to symptoms of nutritional deficiencies (Figure 1). These symptoms often start on the plant's basal leaves and progressively spread to the young foliage. The mild mottling symptoms on the leaves of CCYV-infected plants eventually turn into severe yellowing after two to three weeks. Even while infected fruits did not exhibit symptoms, the viral infection significantly affects the output of zucchini, lowering their market value.

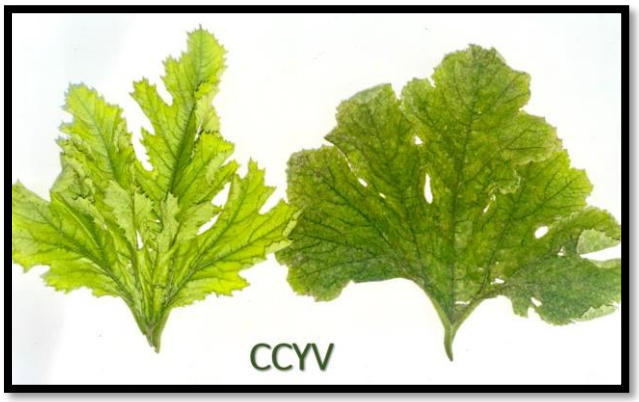


**Figure 1.** Yellowing and chlorosis symptoms of CCYV on zucchini plants

In addition to causing difficult conditions by increasing the chance of virus-carrying vectors living, failure to eradicate weeds that can host-virus diseases within and around the greenhouse gives

vector insects a host opportunity in the summer and winter seasons. Along with the abnormalities in leaf structure brought on by the excess of hormones during the production of zucchini, samples were also taken from plants whose symptoms resembled those of a virus because of the genetic disorder Chimera.

**Molecular results**

Plant samples were tested by RT-PCR and PCR methods in order to determine which of the viruses (CVYV, CYSDV, ToLCNDV, BPYV and CCYV) showing similar yellowing type symptoms infected the plants. In our study, 13 zucchini samples were collected from the cucurbit production area in our country. While being analyzed by one-step RT-PCR method against CYSDV, CVYV, BPYV, which may cause similar yellowing type symptoms in cucurbit growing areas; Molecularly tested by PCR with primers specific for ToLCNDV (Table 1). All samples collected, regardless of symptom pattern, were tested against all the above-mentioned viruses that were the subject of the study.

In RT-PCR and PCR studies, optimization was made for CYSDV, CVYV, BPYV and ToLCNDV, which are known to exist in our country and have positive controls, and total nucleic acids, chemicals and primers were confirmed to work. For the viruses that were the subject of the study, 4 sample was infected with CYSDV were found. The presence of CCYV was further confirmed by amplification of part of genomic segment from RNA2, including the coat protein (CP) gene, which was amplified using primers CCYV-CPF (5'-TCCCGGTGCCAACTGAGACA-3') and CCYV-CPR, 5'-TACGCGCGGCAGAGGAATTT-3'). As a result of all molecular tests, it was determined that the samples were not infected with CVYV, BPYV and ToLCNDV (Figure 2).

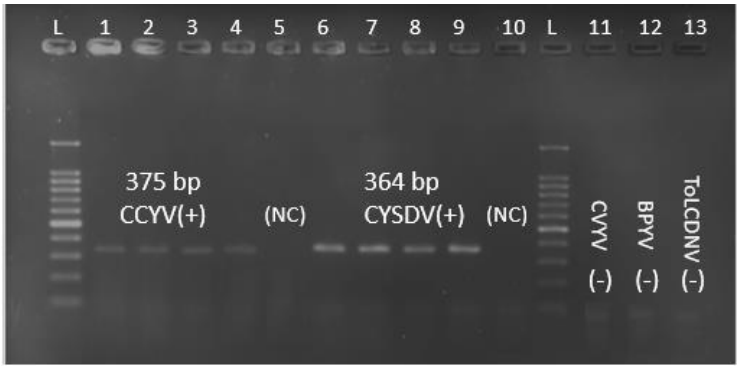


**Figure 2.** PCR and RT-PCR results of CCYV, CYSDV, CVYV, BPYV and ToLCNDV (L: 100bp ladder; 1,2,3,4 CCYV positive samples, 5 negative controls of CCYV; 6,7,8,9 positive samples of CYSDV, 10 negative controls of CYSDV; 11 CVYV negative sample, 12 BPYV negative sample, 13 ToLCNDV negative sample

A DNA ladder indicated that the size of the CCYV PCR product was approximately 375 bp as estimated from the sequence. Tested plants from which a 375 bp DNA fragment was detected were regarded as CCYV-infected.

## DISCCUSSION

A growing class of viruses spread by whiteflies called *Criniviruses* causes annually losses in the billions of dollars around the world (Tzanetakis et al., 2013). *Crinivirus* member CCYV was thought to be localized to Asia, Africa, and the Mediterranean areas of Europe (Bananej et. al., 2013; Orfanidou et al., 2014) till it was recently discovered in the USA (Wintermantel et al. 2019). Further research will be needed to determine CCYV's epidemiology, contribution to disease incidence and severity, and effects on economically significant crops in our country. This is since CCYV, which also occurs in mixed infections in cucurbits with other whitefly-transmitted viruses, can produce symptoms that are quite similar to those of CYSDV (Kavalappara et.al., 2021). In addition, CCYV has been identified in mixed infections with CYSDV with which they share same vectors (Roditakis et al.2009; Orfanidou et al. 2014b). Besides CYSDV, it can show mixed infection with different plant pathogen viruses. These are especially Cucurbit leaf crumple virus (CuLCrV*), Squash leaf curl virus* (SLCV), Beet pseudo-yellows virus (BPYV), Cucumber vein yellowing virus (CVYV), and Tomato leaf curl New Delhi virus (ToLCNDV) (Orfanidou et. al., 2021, Kavalappara et.al., 2021, Abrahamian et. al., 2013, Cuadrado et. al., 2001 and Sáez et. al., 2016)

Cucumber (*Cucumis sativus*) and melon (*C. melo*) plants were found to be affected by a yellowing disease that caused chlorosis and interveinal chlorotic patches on lower leaves in two greenhouses on the Greek island of Rhodes in 2011. In November 2013 in a cucumber greenhouse in Tympaki, Crete, and open field watermelon (*Citrullus lanatus*) plants in Rhodes, similar symptoms were noted (Orfanidou et. al., 2014a). The prevalence of the disease varied from 10% to 40%. The Cucurbit yellow stunting disorder virus (CYSDV), the Beet pseudo-yellows virus (BPYV), the recently described *Cucurbit chlorotic yellows virus* (CCYV), which infects cucurbits in Japan, and the aphid transmitted *Polerovirus* (family *Luteoviridae*) Cucurbit aphid-borne yellows virus (CABYV) were all responsible for the similar symptoms that were observed. In almost most countries, similar problems with this viral agent have been recorded (Orfanidou et. al., 2014a, Bananej et. al., 2013, Al-Saleh et. al., 2015, Amer, 2015, Bananej et. al., 2013, Gu et., 2013 and Hamed et. al., 2011). In our study, we detected mixed infections with CYSDV in 4 samples. We have determined that the symptoms seen on the plant are mixed with other cucurbit viruses and that CCYV is constantly ignored, both in farmer production greenhouses and in commercial companies. For this reason, we predict that the breeding programs used to generate resistant cultivars will encounter significant difficulties as a result of this neglect. Therefore, the importance of accurate and rapid detection of the viral agent becomes evident once again.

Arable weeds from several fields in Rhodes were collected for further investigations. CCYV was detected in 13 species belonging to families in 13.2% of the material collected, and the bulk of the infected weeds were started gathering in November without cucurbits (Orfanidou et al., 2017). Most of these weeds are common in our country; *Amaranthus blitoides, Amaranthus retroflexus, Sonchus*

*oleraceus, Heliotropium europaeum, Capsella bursa-pastoris, Sysibrium sp., Capparis spinosa, Chenopodium albüm, Convolvulus arvensis, Ecballium elaterium, Medicago sativa*, *Malva sylvestrisa* and *Tribulus terrestris* (Orfanidou et al., 2017). Arable weeds are widely known to play a significant part in plant virus epidemiology. Particularly for viruses that are not transmitted through seeds, like *Criniviruses*, they play a critical role in virus dissemination and overwintering. As a result, weed species that are crucial, particularly when the primary crop is absent, have been identified as *Crinivirus* hosts (Boubourakas et al., 2006; Kil et al., 2015; Orfanidouet al., 2014b; Tugume et al., 2016 and Gyoutoku et. al., 2009). Additionally, *Ecballium elaterium* showed a high frequency of CCYV detection, with nearly all the plants exhibiting yellowing symptoms. The effectiveness of vector-mediated viral transmission is a key factor in virus epidemiology. Whitefly vectors serve a particular role in the spread and dominance of *Criniviruses*, which are neither mechanically nor seed transferred (Tzanetakis et al. 2013, Orfanidou et al. 2017).

These results emphasized that although yellowing diseases (CYSDV and CYVV) seen in cucurbits are common in cucurbits in Türkiye, there is another viral disease that has been ignored in addition to these viruses. Given that the infection rate can reach 40% as observed in other countries and that it is sometimes confused for plant nutrient deficiencies, this viral agent poses a serious risk in places where cucurbits are cultivated (Orfanidou et. al., 2014a). Therefore, not ignoring this factor, accurate and fast detection is very important in cucurbit production areas.

## Conclusion

As a result, attention should be paid to whitefly populations in greenhouses and open areas where cucurbits are grown, and precautions should be taken with chemical control when necessary. Major diseases such as CYSDV, CVYV, ToLCNDV can be noticed when the virus abundances that cause yellowing-type symptoms in cucurbits and are carried by whiteflies are examined, and therefore CCYV disease is overlooked. Virus diseases, which are diagnosed with molecular analyzes and cause similar yellowing type symptoms, must be distinguished in important subjects such as resistance studies.

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