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Research Article

Investigation of Virus and Viroid Diseases in Cucurbits

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Abstract: Cucurbita species are economically important vegetables, with Türkiye ranking second in the world for gherkin and cucumber production and sixth for pumpkin and squash production. However, diseases caused by viruses are a threat to global cucurbit production. Due to global warming, changes in agricultural practices, and advancements in virus detection, the number of viruses infecting cucurbits has significantly increased in recent years. In this study, to identify causal viruses (potyviruses and begomoviruses) and viroids (pospiviroids) that induced symptoms on cucurbits, leaves exhibiting virus-like symptoms were surveyed and collected from cultivation areas in Türkiye from July to September 2024, and subjected to detection of causal agents. A total of 150 plant samples were collected from three locations, with plants randomly selected and viral infection symptoms observed. RT-PCR assays for potyviruses identified watermelon mosaic virus (WMV) in 30 samples (20%), which included 11 snake melon, 3 pumpkin, and 16 zucchini samples. Additionally, PCR assays for pospiviroids successfully detected pepper chat fruit viroid (PCFVd) in six samples, including zucchini and snake melon. Amplified products of the expected size were sequenced and showed identities of over 95% with PCFVd isolates in NCBI. This study reports for the first time the identification of zucchini and snake melon as natural hosts of PCFVd.

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1. Introduction

Cucurbita species are among the 10 leading vegetable species (Ferriol and Pico, 2008). Türkiye is the world's second-largest producer of gherkins and cucumbers, with a production of 1.9 million tons. Additionally, it ranked sixth in the world for pumpkin, squash, and gourd production, with an estimated 744.30 tons produced (FAO, 2022). Cucurbit-producing countries must enhance production quality and quantity due to their economic significance. Cucurbits are threatened by various pathogens, including viruses, which vary greatly from country to country. In terms of changes in the viruses that infect cucurbit crops over the years, there were reports that there have been 23 different viruses in 1980, 55 in 2003, 70 in 2014, and 96 in 2023 (Lecoq, 2003; Lecoq and Katis, 2014; Ali, 2023). Climate change, particularly global warming, has led to an expansion in the distribution and population density of insect vectors, such as aphids and whiteflies, which are responsible for transmitting many plant viruses. In addition, intensified agricultural practices, including monoculture cultivation and longer cropping cycles

have created favourable conditions for viruses to emerge and spread. Moreover, advancements in molecular diagnostic tools, including high-throughput sequencing (HTS) and real-time PCR, have markedly increased the sensitivity, specificity, and throughput of virus detection, enabling the identification of previously unrecognized viral species. Cucurbit crops are infected by economically important viruses that belong to different families, such as Geminiviridae, Potyviridae, Bromoviridae, and Luteoviridae (Adams et al., 2012; Lecoq and Desbiez, 2012). The genus *Potyvirus* is the largest genus of the family Potyviridae (Berger et al., 2005). Some of the viruses are quite widespread and cause significant yield losses, while others cause infection in specific crops in limited geographical areas and do not cause significant economic damage. At least twenty potyviruses have been identified as significant threats to cucurbit crop production in the Mediterranean region (Sastry et al., 2019; Desbiez et al., 2020). Among these, zucchini yellow mosaic virus (ZYMV), papaya ringspot virus (PRSV) and watermelon mosaic virus (WMV) are the most common and economically important pathogens infecting cucurbits (Lecoq et al., 2001; Sharma et al., 2013). Potyviruses are efficiently transmitted in a non-persistent manner by many different aphid species and mechanically through plant-to-plant contact or pruning tools (Fauquet et al., 2005; Mishra et al., 2013). Potyviruses have a single-stranded, positive-sense RNA genome of approximately 10 kb characterised a 5' untranslated region (UTR), a single major open reading frame (ORF), and a 3' UTR ending with a poly adenine (Poly A) tail (Sharma et al., 2013). The *Potyvirus* genus encompasses over 180 species, yet only a limited number have been fully sequenced. While *Potyvirus* species exhibit considerable similarity in their genome organization, they display substantial genetic diversity at the nucleotide sequence level (Zhao et al., 2011). The host range of most *Potyvirus* species is relatively narrow; however, bean yellow mosaic virus, turnip mosaic virus, and WMV represent notable exceptions, each demonstrating the ability to infect a minimum of 12 plant families (Moury and Desbiez, 2020).

The family Geminiviridae is one of the largest family with circular single-stranded DNA genomes encapsidated in icosahedral particles (Zerbini et al., 2017). A large number of plant viruses that cause infection in economically important plants are included in this group. The genus *Begomovirus* is included in the family Geminiviridae and contains more than 320 species, accounting for approximately 88% of the total species in this family (Devendran et al., 2022). Begomoviruses are a group of plant viruses that are very important in crops due to their significant effects on agriculture and food production. They have been reported to be widespread in many regions of the world and are important for both developed and developing countries as they threaten the cultivation of economically important crops (Malathi et al., 2017). Begomoviruses, which primarily infect dicotyledonous plants, possess a single-stranded DNA (ssDNA) genome that may be organized either as a monopartite (DNA-A, approximately 2.8 kb) or bipartite (DNA-A and DNA-B, each approximately 2.5–2.7 kb) (Kumar, 2019). These viruses are persistently and non-propagatively transmitted by *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Thompson, 2011). Tomato leaf curl New Delhi virus (ToLCNDV) is a member of *Begomovirus*, containing two circular single-stranded DNA molecules of about 2.5–2.7 kb, referred to as DNA-A and DNA-B (Moriones et al., 2017). ToLCNDV causes major yield reductions in a variety of crops belonging to the Solanaceae and Cucurbitaceae families (Hussain et al., 2004; Lopez et al., 2015). The presence of the virus has been increasing every year and is reported to cause significant economic losses in cucurbit production in many countries (Panno et al., 2016; Venkataravanappa et al., 2020; Siskos et al., 2022). Due to the wide host range of begomoviruses, their high recombination ability and effective transmission by their vector, and their synergistic interaction with different viruses in the host plant, they have a high risk of spreading over large areas in production areas in a short time and causing epidemics. Therefore, identifying the most commonly occurring and damaging viruses is critical for recommending management strategies.

Viroids are classified into two families: Pospiviroidae and Avsunviroidae, with more than 30 species identified worldwide (Di Serio et al., 2017; Matsushita et al., 2018). Among these viroid species, hop stunt viroid (HSVd) has been reported only in cucumbers under natural conditions (Sano, 2003; Lemmetty et al., 2011). Hence, the knowledge of viroids infecting cucurbit plants is limited. The present aimed to gain insight into the presence and evaluate the coexistence of viruses (potyviruses and begomoviruses) and viroids (pospiviroids) in the main cucurbit production areas of Türkiye.

2. Material and Methods

2.1. Field surveys and sampling

A survey was conducted on screening of leaves and fruits of different Cucurbitaceae species in the cities of Hatay, Kahramanmaraş, and Kayseri, Türkiye, from July to September 2024. All samples, including 45 snake melon, 45 pumpkin, and 60 zucchini leaf samples exhibiting virus-like symptoms such as mosaic, yellowing, mottle, leaf curling, and vein clearing (Figure 1) were randomly chosen from the fields.

2.2. Extraction of total RNA and total DNA

Following the cetyl trimethyl ammonium bromide (CTAB) method as described by Li et al. (2008), a singular nucleic acid extraction protocol was implemented for both DNA and RNA virus extractions. Briefly, 100 mg of leaf tissue ground in 1 ml of CTAB extraction buffer (100 Mm Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB, and 0.2% β-mercaptoethanol). The samples were transferred to eppendorf tubes and incubated at 65 °C for 15 min, and centrifuged at 10.000xg for 10 min. The supernatant was transferred to a microcentrifuge tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1), and the mixture was centrifuged at 15.000xg for 10 min at room temperature. The top aqueous phase was transferred to a new tube. About 0.7 volume of cold isopropanol was added, and the mixtures were centrifuged for 10 min at 15.000xg to collect the nucleic acid precipitate. The precipitated nucleic acid was washed with 70% cold ethanol. The ethanol was decanted and residual ethanol was removed by drying at room temperature. The pellet was dissolved in 50 µl sterile ddH_2O. The amount and quality of the RNA and DNA were assessed using a NanoDrop spectrophotometer (ThermoFisher Scientific, USA).

2.3. Complementary DNA synthesis and polymerase chain reaction

The complementary DNA was synthesized from the total RNA extracted as described above, by reverse transcription using the M-MLV RT reverse transcriptase (ThermoFisher Scientific, USA) and random hexamer primers. To identify the species of *Potyvirus* in the cucurbit samples RNA was used in reverse-transcription polymerase chain reaction (RT-PCR) using universal NIb2F and NIb3R primers (Zheng et al., 2010). To search for begomovirus infection, the samples were tested by polymerase chain reaction (PCR) with universal degenerate primers for begomoviruses Begomo-F and Begomo-R (Akhter et al., 2009), and ToLCNDV specific (DNA-A F/R) primer pairs (Kil et al., 2020) (Table 1). Additionally, PCR was performed with universal pospiviroid (Pospi1-F/RE) (Verhoeven et al., 2004) and PCFVd-specific primer (PCF-seq-F/R) (Yanagisawa and Matsushita, 2017) sets to investigate the *Pospiviroid* in cucurbits (Table 1). PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining. PCR products were sequenced in both directions using Sanger sequencing.

2.4. Sequence analysis and construction of phylogenetic trees

Multiple sequence alignments were made using the CLUSTAL W algorithm (Thompson et al., 1994) and BLASTn was performed to check the species homology. For phylogenetic analysis of WMV isolates, a total of 11 isolates were chosen considering different districts and host species. Sequences of WMV (accession numbers: PV582057-PV582067.1) and PCFVd (accession numbers: PQ741727-PQ741732.1) were deposited in GenBank (Supplementary Table 1). The phylogenetic trees were constructed using the MEGA software version 7 (Kumar et al., 2016) based on methods of neighbor-joining and Kimura 2-parameter. Bootstrap resampling (1000 replicates) was used to ensure the reliability of individual nodes in the phylogenetic tree. SDT (sequence demarcation tool) analysis was carried out by using the SDTv1.2 program (Muhire et al., 2014) with defult setting.

Table 1. List of universal and specific primers used to detect Potyvirus, Begomovirus, and Pospiviroid in this study

Primers	Pathogen	Primer Sequence (5'-3')	References
NIb2F NIb3R	*Potyvirus*	GTITGYGTIGAYGAYTTYAAYAA TCIACIACIGTIGAIGGYTGNCC	Zheng et al., 2010
Begomo-F Begomo-R	*Begomovirus*	ACGCGTGCCGTGCTGCTGCTGCCCCCA ACGCGTATGGGCTGYCGAAGTTSAGACG	Akhter et al., 2009
ToLCNDV DNA-A_F ToLCNDV DNA-A_R	*Begomovirus* tomato leaf curl New Delhi virus	GTGATGTACTCCCCTGTGCG ACAAGACAGATGCGTTAAAGGTT	Kil et al., 2020
Pospi1-FW Pospi1-RE	*Pospiviroid*	GGGATCCCCGGGAAAC AGCTTCAGTTGT(T/A)TCCACCGGGT	Verhoeven et al., 2004
PCF-seq-F PCF-seq-R	*Pospiviroid* pepper chat fruit viroid	CCGTCTTCTGACAGGAGTAATCCC ACCCGCACGGCGCTTCTC	Yanagisawa and Matsushita, 2017

3. Results

3.1. Surveys and detection of watermelon mosaic virus and pepper chat fruit viroid by polymerase chain reaction

A total of 150 plant samples were collected from 3 locations in Türkiye. Plants were collected randomly, and viral infection symptoms observed were recorded. The symptoms included severe to mild mosaic, especially newly growing apical leaves of pumpkin and snake melon samples showed severe mosaic symptoms (Figure 1). The squash leaf samples showed the symptoms of leaf curling, yellowing, mottling, mosaic, fan leaf appearance and vein clearing (Figure 1).

Figure 1. Field survey observations of virus-like symptoms in cucurbit crops and reverse-transcription polymerase chain reaction products of a) Potyvirus (NIb2F/NIb3R), b) Pospiviroid (Pospi1-RE/FW), c) pepper chat fruit viroid (PCF-seq-F/R).

Each cucurbit leaf sample was screened RT-PCR with NIb2F/NIb3R universal polerovirus primer pairs, resulting in the amplification of the expected 350 bp fragment from 11 snake melon, 3 pumpkin, and 16 zucchini samples (Figure 1a). Sequence analysis of these amplicons confirmed the out of 30 samples (20%) collected from three provinces was infected with watermelon mosaic virus (WMV) (Table 2). In addition, PCRs conducted with universal begomovirus and ToLCNDV-specific primers yielded no DNA amplification products.

Table 2. Sample numbers for each species, and numbers of infected samples by watermelon mosaic virus and pepper chat fruit viroid as assessed by reverse-transcription polymerase chain reaction

Viral pathogen	Snake melon	Zucchini	Pumpkin	Infected sample /Total number of sample
Watermelon mosaic virus	11/45	16/60	3/45	30/150
Pepper chat fruit viroid	2/40	4/50	Not detected	6/150

Moreover, RT-PCR was performed on the samples using the universal primer sets Pospi1-RE/FW (Verhoeven et al., 2004). Six samples produced amplicons of 189 bp with Pospi1-FW and Pospi1-RE primer pairs, which were then directly sequenced (Figure 1b). Sequencing of the amplicons showed identities of more than 96% identity with PCFVd isolates (KC762954.1, MW422292.1, and MW422290.1) in NCBI. To verify the presence of PCFVd in field samples, RT-PCR was performed using two pairs of PCFVd-specific primers (PCF-seq-F/R) (Yanagisawa and Matsushita, 2017). PCR products with the expected size (Figure 1c), obtained from 2 snake melon and 4 zucchini samples, were purified from agarose gel by the use of a Qiaquick gel extraction kit and subjected to bidirectional Sanger sequencing to confirm the presence of the viroid.

3.2. Sequence and phylogenetic analyses

Pairwise nucleotide sequence identities of the eleven WMV isolates obtained in the present study, determined by CLUSTAL W alignment, ranged from 91% to 100% (Figure 2a), whereas they shared the maximum nucleotide sequence identities of 91-99% with other isolates of WMV. Phylogenetic analysis of WMV isolates showed that sequences were grouped into several clusters (Figure 2b). Three of the WMV isolates (WMV21, WMV38, WMV49) were obtained from zucchini clustered with isolates from Iraq and Iran (MT780536.1, MT780537.1), France (EU660581.1, EU660586.1, JF273463.1, JF273467.1) and Spain (MH469650.1). Whereas four isolates (WMV9, WMV15, WMV18, WMV23), obtained from snake melon and zucchini, were clustered into an independent clade, shared the highest nucleotide identities at 95% to reference isolates. The other four isolates (WMV2, WMV19, WMV26, WMV44), obtained from zucchini and pumpkin, clustered separate clade the others with 99% bootstrap value (Figure 2b). Phylogenetic analysis show that the clustering WMV isolates were not associated with geographic origins or host species.

Pairwise sequence identity matrix from nucleotide (Figure 3a) sequences generated using the SDT 1.2 software, and six PCFVd-cucurbit isolates obtained in the present study were 100% identical to each other. The phylogenetic analysis of the PCFVd-cucurbit isolates was done to infer the relationship of the current isolates with the previously reported isolates in NCBI. Six PCFVd-cucurbit sequences were analysed, and the sequences were retrieved from the NCBI after BLASTn of these sequences. The BLASTn search of the NCBI database revealed high sequence identities (95% to 99.71%) with previously reported PCFVd isolates from different geographic regions. Furthermore, phylogenetic analysis showed that the six PCFVd-cucurbit isolates clustered together with isolates from tomato (KC762953.1, KC762954.1, MW422292.1) from Australia (originated in Israel) and the Netherlands, as well as isolates from pepper (MW012406.1, MW012415.1, MW422288.1) from Vietnam and the Netherlands (Figure 3b). Notably, this is the first report of PCFVd in zucchini and snake melon worldwide.

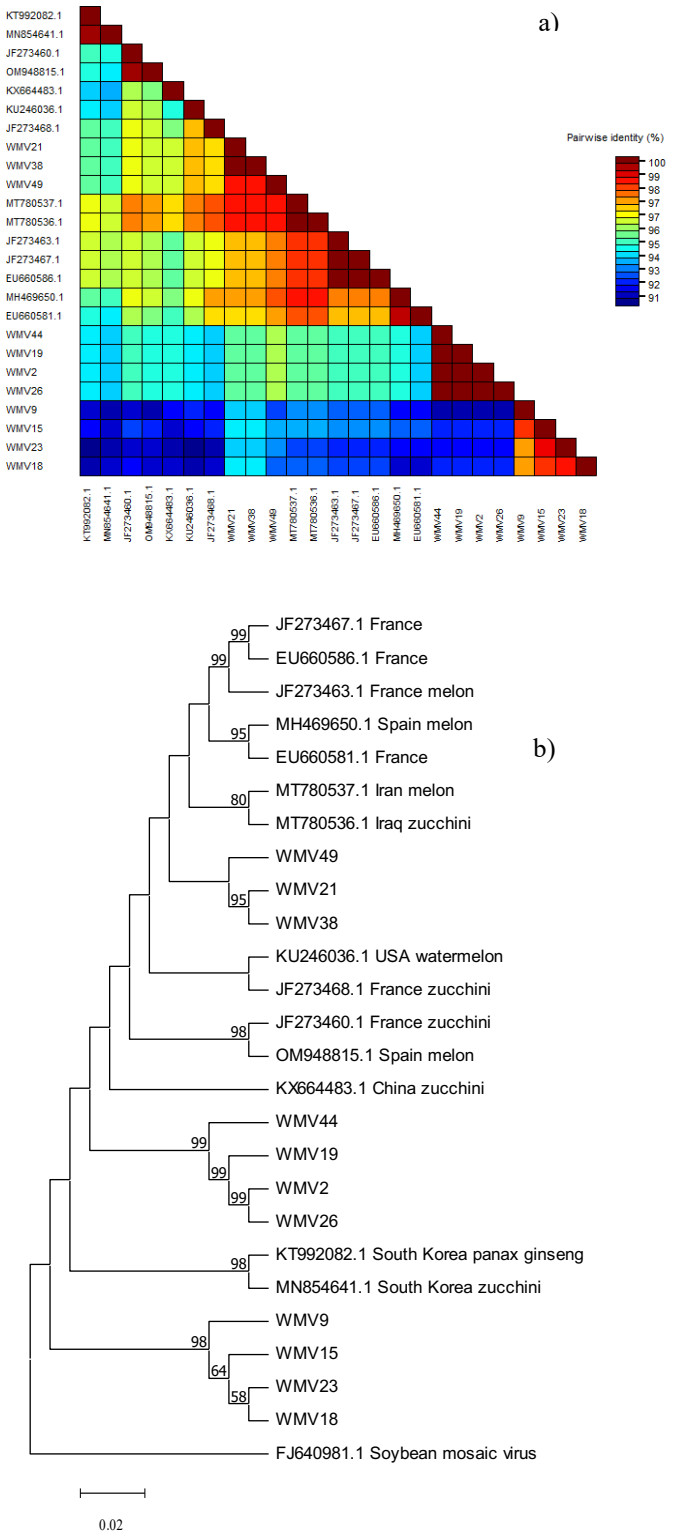

Figure 2. Sequence comparison and phylogenetic analysis of watermelon mosaic virus (WMV) isolates. The pairwise identity scores of WMV were generated using Sequence Demarcation Tool version 1.2 software (a). Phylogenetic tree showing relationship of WMV isolates in this study and from NCBI database (b). Soybean mosaic virus (FJ6440981.1) was used as the outgroup.

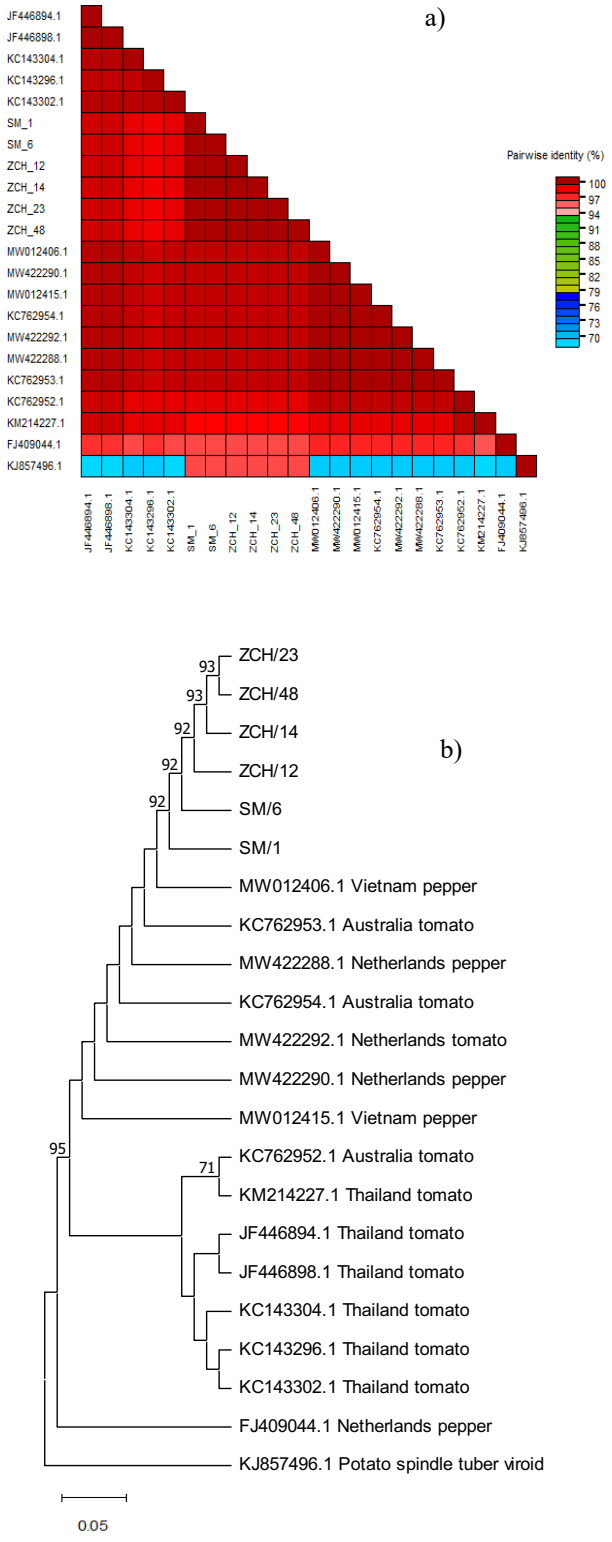

Figure 3. Sequence comparison and phylogenetic analysis of pepper chat fruit viroid (PCFVd) isolates. The pairwise identity scores of PCFVd isolates were generated using Sequence Demarcation Tool version 1.2 software (a). Phylogenetic tree showing relationship of PCFVd isolates in this study and from NCBI database (b). Potato spindle tuber viroid (KJ857496.1) was used as the outgroup.

4. Discussion

WMV, alongside with zucchini yellow mosaic virus (ZYMV) and papaya ringspot virus (PRSV), is one of the most destructive potyviruses which cause serious yield losses to cucurbit crops around the world (De Moya-Ruiz et al., 2023; Sharma, 2023). WMV have been commonly recorded in squash, watermelon, melon, pumpkin (Moreno et al., 2004; Yeşil, 2019; Pérez-de-Castro et al., 2020) and, has even been detected in snake melons and bottle gourds for the first time (Güller et al., 2024). Consistent with previous studies, we also detected WMV infection in 11 snake melon samples. Moreover, WMV is not seed-borne in cucurbits but can be transmitted non-persistently by at least 35 species of aphids, allowing the virus to spread rapidly among cucurbit plants. The geographical distribution of WMV is influenced by a range of factors, including the ecology of aphid vectors, the presence of wild plant host species, and the impacts of climate change (De Moya-Ruiz et al., 2023). Specifically in the Mediterranean basin, WMV has been reported in most countries as one of the dominant cucurbit viruses (Moreno et al., 2004; Pérez-de-Castro et al., 2020; De Moya-Ruiz et al., 2021). The high diversity of host plants and weeds in production areas contributes to the increased prevalence and infection rates of viral diseases (McLeish et al., 2017). Climate change, in particular, can extend growing seasons, allowing for more generations of vectors and increased opportunities for virus transmission. The interaction between aphid populations and the virus can lead to variations in the prevalence of WMV across different regions and cropping systems. Additionally, the global trade of vegetatively propagated plants has the potential to accelerate the spread of the virus, thereby increasing the molecular diversity within its population (Desbiez et al., 2020). Utilizing resistant cultivars is a recommended strategy for managing WMV, as it has been shown to effectively reduce both virus titers and symptom severity across various isolates (Díaz-Pendón et al., 2005). Besides, the use of silver reflective plastic mulches at planting is an effective management strategy to delay WMV infection in young cucurbit plants. These mulches repel aphid vectors by reflecting light, thereby reducing early-season virus transmission and promoting healthy initial plant growth until canopy closure diminishes their efficacy.

Whitefly-transmitted begomovirus infections on cucurbits has been recognized as an emerging disease in various regions worldwide, resulting in significant yield losses (Juárez et al., 2014; Yazdani-Khameneh et al., 2016; Cai et al., 2023; Troiano and Parrella, 2023). Fidan et al. (2023) reported the presence of ToLCNDV in cucurbits grown in greenhouses in Türkiye. However, in the present study, despite the observation of symptomatic plants exhibiting leaf curling, yellowing, and stunting, ToLCNDV was not detected in cucurbits. Pepper chat fruit viroid, *Pospiviroid parvicapsici*, belongs to the genus *Pospiviroid* of the family Pospiviroidae (Walker et al., 2021). PCFVd was initially identified as a novel pospiviroid species that infected sweet peppers (*Capsicum annuum* L.) (Verhoeven et al., 2009), later reported in tomato and pepper cultivars in Thailand, Vietnam and Australia (Yanagisawa and Matsushita, 2017; Reanwarakorn et al., 2011; Keyata et al., 2024). This is the first report of cucurbits being a natural host of PCFVd worldwide.

Conclusion

Cultivated under open field conditions in Türkiye indicating a natural occurrence of PCFVd in zucchini and snake melon. PCFVd was initially found in pepper and subsequently in tomato crops in various regions around the world. The detection of PCFVd in zucchini and snake melon represents a novel finding. The host range of viroids is narrow, but in recent years there have been studies showing that the host range of some agents has expanded. In most cases the origin of these infections remained unknown. The presence of WMV and PCFVd highlights the ongoing viral threats to cucurbit production, while the absence of begomoviruses in the surveyed samples is noteworthy.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The author declares that there are no conflicts of interest.

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Author Contributions

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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