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RESEARCH ARTICLE

# Occurrence and Molecular Characterization of Some Economically Relevant Cucurbit Viruses in Malatya, Turkey

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**ABSTRACT**

Field surveys were conducted from 2016 to 2017 to detect and determine the prevalence of viruses in the major cucurbit growing areas of Malatya. A total of 162, namely 110 melon, 25 pumpkin and 27 watermelon leaf samples were collected from Arapgir, Arguvan and Battalgazi districts from symptomatic and non-symptomatic cucurbit plants. Using molecular methods, the plant samples were tested against four viruses, including Cucumber mosaic cucumovirus (CMV), Zucchini yellow mosaic potyvirus (ZYMV), Watermelon mosaic potyvirus (WMV) and Squash mosaic comovirus (SqMV). Results of RT-PCR tests revealed that none of the samples collected from Arapgir and Arguvan reacted positive against surveyed viruses. However, CMV infection was found in 1 cucumber, ZYMV in 5 pumpkin and WMV in 6 watermelon samples in Battalgazi district. The nucleotide sequences of partial coat protein (CP) genes of ZYMV-Malatya and WMV-Malatya isolates were determined. Nucleotide sequence comparisons revealed 95-100% similarity of ZYMV-Malatya isolate with other isolates of ZYMV reported worldwide with no mutation site on CP gene. However, WMV-Malatya isolate exhibited 94-99% similarity with other WMV sequences reported worldwide consisting a unique mutation site on CP gene sequence. These isolates are recorded to the Gene Bank with the accession numbers MT186266 (Zucchini yellow mosaic virus isolate, Malatya ZYMV1) and MT186267 (Watermelon mosaic virus isolate Malatya WMV1).

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## Introduction

The species in the Cucurbitaceae family are the most consumed vegetables. It is one of families of the Angiosperms (besides Brassicaceae, Fabaceae, Poaceae, and Solanaceae). Mainland of cucurbits are Central Asia and they are suitable to cultivate in temperate and tropical regions. According to FAO statistics, China is the first producer of cucurbits with 1.237.358,00 ha production areas followed by Turkey with 37.695,00 ha production areas (Anonymous, 2019).

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There are approximately 26 million tons of vegetable production in an area of 1.056.000 ha in Turkey. Nearly 40 % of this production belongs to *Cucurbitacea* family (Anonymous 2018).

The production of the surveyed areas, Arguvan, Arapgir and Battalgazi districts of Malatya, are 7800 da, 7.860 da and 260 da, respectively (Anonymous 2018).

*Zucchini yellow mosaic virus* (ZYMV) belongs to potyvirus genus with about 750 nm long flexuous rods, having a single strand of RNA. The characteristic symptoms of the virus are severe plant stunting, yellow mosaic, shoe stringing, severe malformation, reduction of leaf lamina size, necrosis and deformation of seeds and fruits. The virus is reported in France, Germany, Italy, Israel, Lebanon,

Morocco, Spain, the USA (Lecoq et al. 1983), Egypt (Provvidenti et al. 1984). The experimental host range includes members of the families Labiatae, Leguminosae, Aizoaceae, Scrophulariaceae, Chenopodiaceae, Amaranthaceae, Compositae, Ranunculaceae, Cucurbitaceae, Solanaceae and Umbelliferae (Lisa et al. 1981, Lecoq et al. 1981). Although it is not transmitted by seed (Lecoq et al. 1981), it is transmitted by *Aphis citricola* (Purcifull et al. 1984), *A. gossypii*, *Myzus persicae* (Lisa et al. 1981, Lecoq et al. 1981,) and *Macrosiphum euphorbiae* non-persistently.

*Cucumber mosaic virus* (CMV) belongs to *Bromoviridae* family and is the type member of the *Cucumovirus* genus having a very broad host range (Palukaitis and Arenal 2003). Infecting more than 1.200 plant species (Douine et al. 1979, Kaper and Water worth 1981), CMV has the widest host range among plant viruses. The single-stranded RNA of the virus has three functional pieces. The virus is distributed world-wide and it is transmitted by numerous species of aphid non-persistently (Palukaitis et al. 1992). It shows a variety of symptoms on different hosts (Lee et al. 2008, Kaper and Water worth 1981, Martelli and Russo 1985, Jeon et al. 2006, Lee et al. 2007, Oh et al. 2008, Choi et al. 2004). It causes mosaic symptoms on cucumber and many other cucurbits, fern leaf on tomato, mosaic on celery, blight on spinach and mosaics on many dicotyledonous and monocotyledonous plants, ornamentals, trees, shrubs and weeds. Also it causes breaking of flowers and dwarf of the plants. CMV is transmitted by at least 10 species of *Cuscuta*. By nucleotide sequence similarity, its trains fell into two major groups; and subgroup I has been further divided into two subgroups phylogenetically (Chaumpluk et al. 1996, Roossinck et al. 2003, Aramburu et al. 2007).

*Watermelon mosaic virus, being* a member of *Potyvirus* genus in *Potyviridae* family, causes infections in most cucurbits and some legumes. The virus has a wide host range from 27 different families and more than 170 different plant species (Asad et al. 2006). The main symptoms are mottling and mosaic, but they can vary depending on some other features like the host species, the plant cultivar, virus strain and environmental factors. Other symptoms that can be observed on diseased plants are mottling, blisters, stunt and leaf distortion in cucumber, muskmelon, pumpkin, squash, and watermelon, and mottle and chlorosis in pea and lupin (Inouye 1964).

## Materials and Methods

### *Collecting Cucurbit Samples*

Surveys of open field cucurbit crops including melon (*Cucumis melo*), squash (*C. maxima*), zucchini (*Cucurbita pepo*), cucumber (*C. sativus*) and watermelon (*Citrullus lanatus*) were conducted during 2016 and 2017 in three cucurbit growing districts of Malatya province that included Arapgir, Arguvan and Battalgazi. During the surveys a total of 162 cucurbit planta with typical viral symptoms like yellow mosaic, reduction of the leaf lamina, necrosis, malformation, stunting, blisters,and without symptoms were sampled randomly from the shoot apex of plants throughout the field in each district (Table 1).

**Table 1.** Number of cucurbit samples collected from surveyed locations

| Arguvan Province | Number of samples collected | Arapgir Province | Number of samples collected | Battalgazi Province | Number of samples collected |
|---|---|---|---|---|---|
| Bahçeli | 12 | Cömertli | 8 | Merkez | 10 |
| Çiftlik | 12 | Deregezen | 11 | Kampüs | 2 |
| İsa köy | 10 | Hezenek | 10 | | |
| Karahüyük | 7 | Kazanç | 9 | | |
| Morhamam | 10 | Kılıçlı | 7 | | |
| Narmikan | 9 | Pirali | 9 | | |
| Yazıbaşı | 7 | Sinikli | 11 | | |
| Yolağzı | 9 | Yolçatı | 3 | | |
| | | Yukarı ulupınar | 6 | | |
| | 76 | | 74 | | 12 |
| TOTAL | 162 | | | | |

### *Total RNA Extraction*

Total RNA preparations were conducted according to Foissac et al. (2001). RNA of an asymptomatic cucumber plant was served as the negative control. A leaf tissue of hundred milligrams was grounded in 1 ml grinding buffer and the homogenate was cleared by centrifuging 13.000 g. for 5 minutes. Then, 10% Sodium lauryl sarcosyl solution (100 µl) was added to 500 µl of the homogenate and incubated 10 min at 70°C by intermittent shaking. The extract was incubated 5 minutes on ice. Following centrifugation at 14.000 g for 10 min, supernatant (300 µl) was transferred to a new centrifuge tube and 300 µl of 6M sodium iodide, 25 µl resuspended silica and 150 µl of ethanol was added. The mixture was shaked intermittently and kept 10 minutes at room temperature. Tubes were centrifuged at 6000 g for 1 minute and the supernatant was poured and the pellet was washed twice with 500 µl washing buffer. Silica pellet was then dried briefly and nucleic acids were diluted with 100 µl PCR grade water and incubated for 4 minutes at 70°C on thermoblock. After centrifugation at 14.000 g for 3 minutes, the supernatant was transferred to a new tube and stored at -20° until use.

### *RT-PCR Tests*

Reverse transcription was carried out using virus-specific antisense primers with a total volume of 25 µl containing 2 µl total RNA, 1 µl 100 nmol of each primer, and 7 µl RNase free water, 5 µl of 5X RT buffer, 5 µl of dNTP (each 2.5 mM) mixture, 0.5 µl of RNase inhibitor and 1 µl of MMLV reverse transcriptase enzyme.

PCR tests were performed according to Wang et al. (2010) with slight modifications. A 25 µL of PCR reaction volume was contained 2 µL of RT product, 2.5 µL of 10 x PCR reaction buffer, 2 mM MgCl2, 2 µL of dNTP mixture (each at 2.5 mM), 1µL of each primer and 0.5 U of Taq DNA

polymerase enzyme. PCR condition for reaction was as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 45 s, annealing at 46°C for 45s, extension of 1 m at 72°C and a final extension for 10 min at 72°C. Before visualizing with a UV trans-illuminator the amplified DNA was electrophoresed on 2 % agarose gel and stained with flourescent nucleic acid staining solution. A 100 bp DNA ladder was used as marker (100bp DNA ladder, Thermo Scientific). The primers used for the viruses are given on Table 2.

**Table 2.** Primers used in PCR tests

| Virus | Accession number | Sequence | Band size (bp) | Annealing temperature |
|---|---|---|---|---|
| CMV-F | SEQ ID NO 1 | ATTGGTCGTCCCACTCTT | 293 | 51.5 |
| CMV-R | SEQ ID NO 2 | TCGCCAAACATAGCAGAG | | 52.3 |
| SqMV-F | SEQ ID NO 3 | AGGCACATTTCGCAGTTC | 354 | 53.7 |
| SqMV-R | SEQ ID NO 4 | CGATGGTTGCCTTTATGT | 354 | 51.1 |
| WMV-F | SEQ ID NO 7 | CCAGTGGCAAAGGTGATA | 485 | 52.7 |
| WMV-R | SEQ ID NO 8 | TGCTGCGTCTGAGAAATG | 485 | 55.4 |
| ZYMV-F | SEQ ID NO 9 | GGAGCGGAAACAAGTGAA | 542 | 53.7 |
| ZYMV-R | SEQ ID NO 10 | ACCTGCTCATTCCCATCC | 542 | 54.6 |

### *Sequence Analysis*

Amplified PCR products were sequenced by a commercial firm via using the dideoxy chain termination reaction (Sanger et al. 1977).

## Results and Discussion

### *RT-PCR Tests*

None of the 110 melon samples collected from Arguvan and Arapgir districts were reacted positive against viruses tested. However, 1 cucumber, 5 pumpkin and 6 watermelon samples taken from Battalgazi district were tested positive against CMV, ZYMV and WMV, respectively. One of each isolate was selected randomly and partial viral genomes of detected viruses were successfully amplified by RT-PCR. The DNA fragments lengths were in expected sizeof 293, 485 and 542 for CMV, WMV and ZYMV, respectively (data not shown). Leaf and fruit symptoms of virus infected samples are shown in Figure 1.

**Figure 1.** Leaf malformation, mosaics and fruit blisters on ZYMV infected Cucurbits.

### *Analysis of PCR Products*

BLASTn search for sequence similarity revealed that ZYMV isolate (Accession no. MT186266) showed 100 % similarity with German (accession number: AJ420019.1), Austrian (accession number: AJ420014.1) and Indian (accession number: KT778297.1) isolates (Table 3).

**Table 3.**The similarity rates of ZYMV isolate with the other ZYMV isolates in the world

| Country | Acession number | Similarity |
|---|---|---|
| Persia | JN183062.1 | % 99 |
| Syria | AB458595.1 | % 99 |
| Germany | AJ420019.1 | % 100 |
| France | JX310105.1 | % 99 |
| Jordan | EU999757.1 | % 99 |
| Serbia | JX262133.1 | % 99 |
| Austria | AJ420014.1 | % 100 |
| India | KT778297.1 | % 100 |
| Venezuela | JX310107.1 | % 97 |
| Poland | EU561043.1 | % 96 |
| China | AY611021.1 | % 95 |
| Serbia | JX262120.1 | % 98 |
| Slovakia | DQ124239.1 | % 98 |
| Dominique Republic | JX310110.1 | %98 |
| Japan | AB004641.1 | % 98 |

The nucleotide sequences of ZYMV isolate were aligned and compared with the other world isolates.

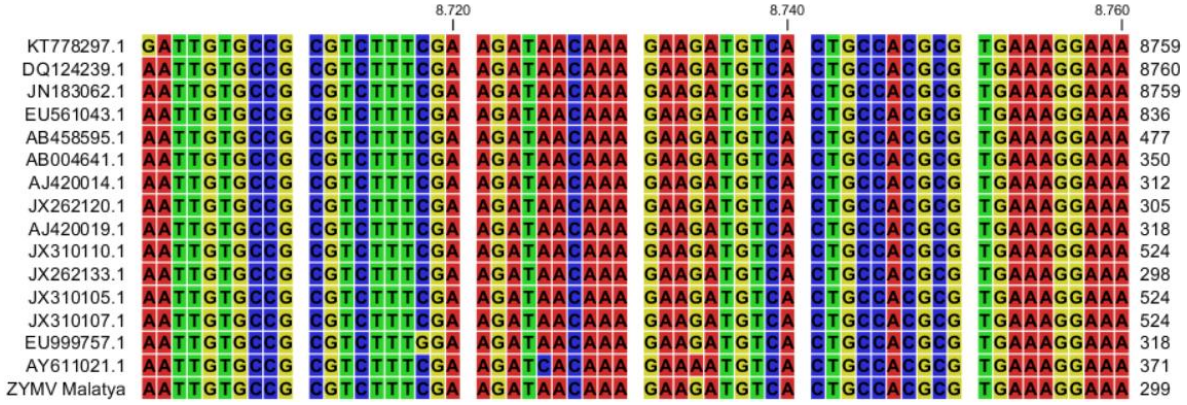


**Figure 2.** Multiple alignment of nucleotide sequences of ZYMV-Malatya and ZYMV world isolates exhibiting a high level of similarity

A phylogenetic tree based on the CP sequence of ZYMV isolate was created via CLC Main Workbench (Qiagen) **software and is shown in Figure 3.**

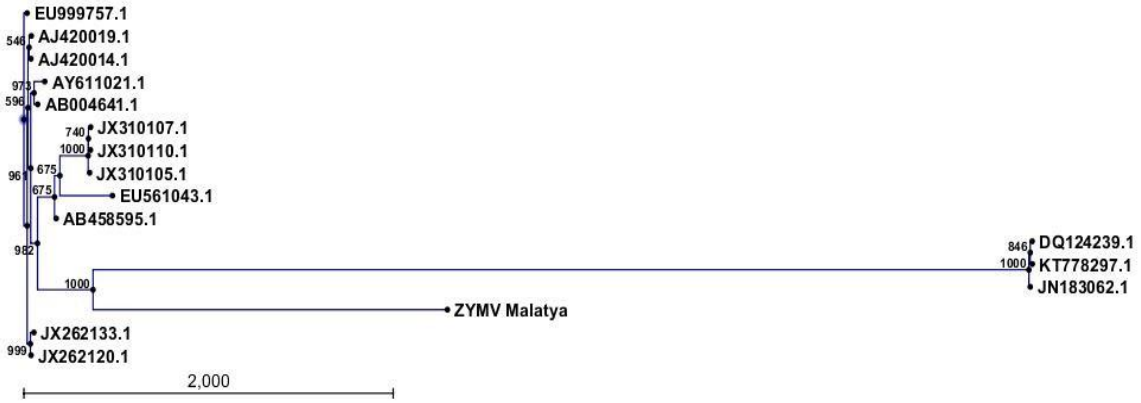


**Figure 3.** The phylogenetic tree of ZYMV isolate

Multiple alignments revealed that WMV-Malatya isolate showed 95-99 % similarity with the WMV world isolates retrieved from GenBank. WMV-Malatya isolate (Accession no. MT186267) exhibited a high degree of similarity with Chinese isolates (accession number: KM527440 and AY464948.1, Table 4).

**Table 4.** The similarity rates of WMV isolate with the other WMV isolates in the world

| Country | Accession number | Similarity (%) |
|---|---|---|
| China | KM527440.1 | 99 |
| Poland | FJ628395.1 | 98 |
| India | KM597071.1 | 95 |
| Persia | GQ421156.1 | 95 |
| Korea | KT992092.1 | 94 |
| Ukraine | KJ461321.1 | 94 |
| France | EU660581.1 | 95 |
| Turkey | KF021300.1 | 94 |
| Serbia | JX262115.1 | 94 |
| China | AY464948.1 | 99 |
| Korea | KT992086.1 | 98 |
| Korea | KT992090.1 | 98 |
| China | KM527488.1 | 97 |
| Persia | JN166706.1 | 95 |
| Spain | AJ579497.1 | 95 |

The nucleotide sequences of WMV-Malatya isolate exhibited a distinctive mutation at nucleotide positions of 9318, which distinguished the Turkish WMV-Malatya isolate from all previously reported WMV world isolates (Figure 4).

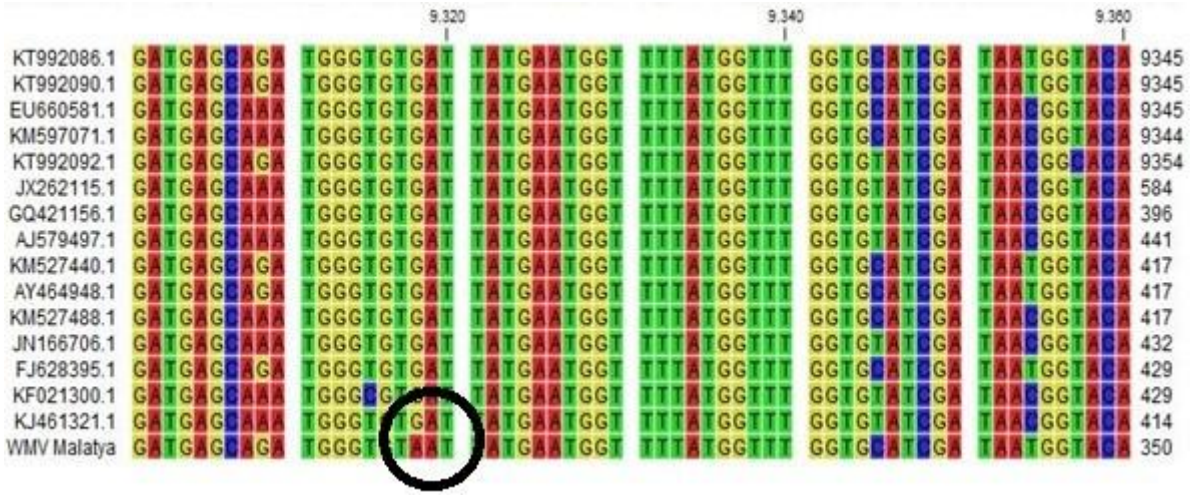


**Figure 4.** The mutation site of WMV-Malatya isolate identified by CLC Main Workbench 8 program

A phylogenetic tree based on the CP sequence of WMV-Malatya created via CLC Main Workbench (Qiagen) software and WMV world isolates has been shown in Figure 5.

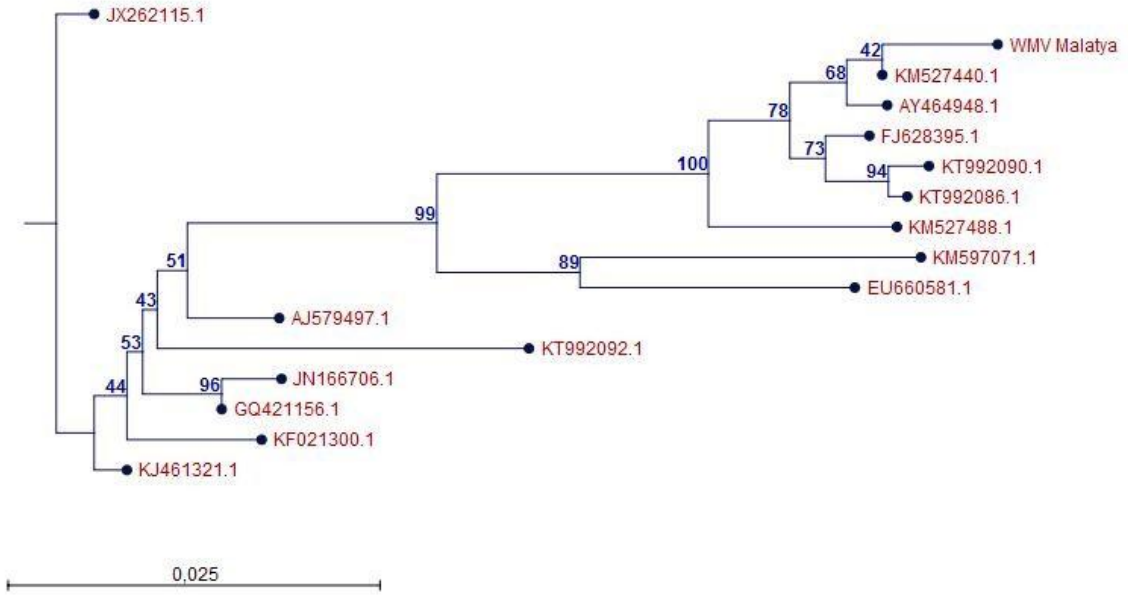


**Figure 5.** The phylogenetic tree of WMV

The present study is the first study reporting viral diseases infecting cucurbits in Malatya province. A total of 162 cucurbit samples were molecularly tested against the most destructive cucurbit viruses (CMV, ZYMV and SqMV) during 2016-2017 growing season in Malatya. The sequence analysis was performed to the PCR products revealed a mutation site in WMV-Malatya isolate whereas no mutation was detected in ZYMV-Malatya isolate.

Ali et al. (2005) determined the complete nucleotide sequence of a Pakistani isolate of WMV (WMV-Pk) and reported that this isolate showed similarity with a French isolate, WMV-Fr with 96% (amino acid) and 94.4% (nucleotide), respectively. They suggested that little differences could be due to a mutation of a common ancestor virus or because of the mutations caused by

different selection pressures in two different agro-ecological zones.

Khanal and Ali (2019) reported the complete genome sequence of ZYMV isolate namely BL-67, isolated from pumpkin during the 2016 growing season in Oklahoma. When compared with the isolates in the Genebank, it showed 83% to 96% nucleotide sequence identity and 86% to 98% amino acid sequence identity. The highest amino acid (98%) and nucleotide (96%) sequence similarities were found with an isolate from South Korea (GenBank accession no. MH042025) and 95% amino acid and 92% nucleotide identities with an USA isolate of ZYMV.

Glassa and Pittnevora (2006) determined the complete nucleotide sequence of a Slovak isolate of ZYMV-Kuchyna. The similarity of the sequences were 90.4–98.8% and 78–98.8% with 12 sequenced ZYMV isolates from the GeneBank at the nucleotide and amino acid levels, respectively. Phylogenetic analysis of the complete capsid protein (CP) sequences form a phylogenetically homogeneous group and they were closely related with more than 50 geographically different ZYMV isolates.

Özer et al. (2012) cloned CP genes of two severe Turkish isolates of ZYMV and determined their complete nucleotide sequences and deduced amino acids. The CP of the two isolates showed a similarity of 93%-98% and 94%-99%, respectively, at the nucleotide level with 23 isolates. When compared with the other isolates from different geographical regions worldwide, ZYMV-Ah isolate was clustered with isolates from Far Eastern countries (Taiwan and Korea) whereas ZYMV-Ad isolate clustered with isolates from Middle Eastern countries (Syria, Israel and Jordan).

Eiras et al. (2004) performed biological, serological and molecular analysis of CMV isolates collected from different plants in Brazil. With ELISA and RT- PCR/RFLP tests, they confirmed that the isolates belong to subgroup I of CMV. The isolates showed 92 to 99% similarity with the subgroup I CMV isolates. Phylogenetic tree with the analyses of the multiple sequence alignment of the nucleotides and the translated amino acid sequences revealed three distinct clusters. And they reported that these results indicate the prevalence of the sub- group I of CMV in Brazil.

Nouri et al. (2014), studied the genetic variation of 32 CMV isolates taken from different parts of the USA. They found out that there is a low nucleotide diversity in the CMV isolates of U.S. Sequence and phylogenetic analyses revealed that CMV subgroup I is predominant in the USA, following mixture of subgroups IA and IB.

Arafati et al. (2013) performed a survey to determine the presence of CMV subgroups during 2011-2012 in Iran. They sequenced coat protein (CP) gene of 19 CMV isolates and found out via phylogenetic analysis that 4 isolates from Khuzestan fell into subgroup IB and the other Iranian isolates in this study are from subgroup IA.

The viruses tested was not detected on melons but they were detected in other cucurbit plants. It is concluded that the main reason of this is that the producers use domestic varieties and they re-use the seed of these cultivars the other year. This data may indicate that these cultivars may have a resistance to the viruses. Therefore, further studies are necessary to determine if there is a resistance against these viruses in the province.

The absence of the viruses tested in melon-growing areas does not mean that it will not be found in the future. For this reason, the producers must always be cautious. In addition, the necessary measures should be taken to prevent the viruses found in pumpkin, cucumber and watermelon. All melon varieties cultivated in these districts should be investigated for their possible resistance genes and should be used as gene source to produce resistant varieties. Breeding programs for disease resistance, using either the traditional genetic methods or engineering approaches based on pathogen-derived sequences, should be perform to determine the genetic variability. Although these viruses are transmitted by aphids, no virus was detected in the melon areas next to other cucurbitaceous plants. Therefore it is necessary to carry out detailed research on the vector potential of aphids. The other viruses infecting melons should be investigated and if there is a virus infection, a map of viruses should be determined. Also, virus-free, resistant and tolerant varieties should be used in order to avoid any viral problems in this region in the future.

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