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Original Article

# Importance and molecular properties of *Cucumber Mosaic Virus* in cucurbit fields of Jahrom, Iran

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## Abstract

*Background & Objectives:* The *Cucumber Mosaic Virus* (CMV) is a worldwide distributed *Cucumovirus*, and one of the important viruses of cucurbits and other agricultural crops, which causes a significant reduction of yield. This study was done to determine some information about the distribution, importance, and molecular characteristics of CMV in cucurbitaceous fields of the Jahrom area.

*Material and Methods:* 147 plant samples suspected of CMV symptoms were collected from leaves of different varieties of cucumber, pumpkin, watermelon, and melons from various farms of cucurbits and cucumber greenhouses of the Jahrom area and were investigated using DAS-ELISA and RT-PCR methods. The PCR products of two-isolates were sequenced from the Ghotbabad and Hormoj villages. These isolates sequences were compared with 19 selected sequences of NCBI. Using *Clustal W* and *Mega6* software, the phylogenetic tree was drawn via the Neighbor-Joining method.

*Results:* 59 samples were detected as CMV infected by DAS-ELISA test. The RT-PCR reaction confirmed the infection. Sequences analysis showed that the two isolates of CMV-JS1 and CMV-JS2 from the Jahrom area with the isolates of M21464.1, AJ242585, AJ866272.1, and L40953.1, respectively from Australia, China, India, and South Africa are located in an individual subgroup.

*Conclusion:* The results of this study showed that there where a significant infection in cucurbit fields of the Jahrom area and may cause significant yield losses. Since the CMV has a wide host range and is transmitted by aphids, hence it is recommended to control weeds as well as vector aphids.

*Keywords:* *Cucurbitaceae,* DAS-ELISA, Phylogenetic analysis.
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Original Article

# اهمیت و ویژگی های مولکولی ویروس موزائیک خیار مزارع جالیزی جهرم

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## چکیده

*سابقه و هدف:* *ویروس موزاییک خیار*(CMV) یک *کوکوموویروس* با انتشار جهانی و یکی از ویروس های مهم کدوئیان و سایر محصولات است که سبب کاهش قابل توجه محصول می گردد. این پژوهش به منظور به دست آوردن اطلاعاتی در مورد انتشار، اهمیت و ویژگی مولکولی CMV در مزارع جالیزی منطقه جهرم انجام شد.

*مواد و روش ها:* ۱۴۷ نمونه گیاهی مشکوک به علایم CMV از برگ های بوته های ارقام مختلف خیار، کدو، هندوانه و خربزه از مزارع مختلف کدوئیان و گلخانه های خیار شهرستان جهرم جمع آوری و با استفاده از آزمون سرولوژیکی الایزا (DAS-ELISA) و آزمون مولکولی RT-PCR مورد بررسی قرار گرفتند. محصولات PCR مربوط به دو جدایه از روستاهای قطب آباد و هورموج تعیین توالی و با ۱۸ توالی NCBI مقایسه شدند. سپس درخت فیلوژنتیکی با کمک نرم افزار های ClustalW و MEGA6 با روش Neighbor-Joining ترسیم گردید.

*یافته ها:* با استفاده از آزمون الیزا ۵۹ نمونه آلوده به CMV تشخیص داده شد. واکنش RT-PCR نیز آلودگی ها را تأیید نمود. آنالیز توالی ها نشان داد که دو جدایه CMV-JS1 و CMV-JS2 شهرستان جهرم همراه با جدایه های M21464.1، AJ242585، AJ866272.1، L40953.1 به ترتیب از استرالیا، چین، هند و آفریقای جنوبی در یک زیر گروه قرار می گیرند.

*نتیجه گیری:* نتایج نشان داد که آلودگی قابل توجهی به ویروس موزائیک خیار در مزارع جالیزی منطقه جهرم وجود دارد که می تواند کاهش محسوس محصول را به دنبال داشته باشد. به دلیل دامنه میزبانی وسیع CMV و امکان انتقال به وسیله شته ها، از این رو توصیه می گردد که علاوه بر کنترل علف های هرز در مزارع مبارزه با شته ها نیز انجام شود.

*واژگان کلیدی:* *کوکوربیتاسه*، DAS-ELISA، آنالیز فیلوژنتیکی.

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## Introduction

The first report of *Cucumber Mosaic Virus* (CMV) was in 1916 as a mosaic disease in cucumber and watermelon plants from the state of Michigan, USA (1). After that, CMV was reported from many countries of the world, especially in tropical and temperate regions. It was identified and reported by different names from other parts of America, Europe, and Africa, and other parts of the world, especially from temperate regions (2). Many epidemics of this virus have reported from around the world, for example, the acute and destructive tomato necrosis in Japan (3), Italy (4, 5) and Spain (6), whitening of tomato leaves in New York, legumes mosaic in southeastern America, melon mosaic in California, greenhouses plants mosaic in Europe (1) and shoestring of leaves of lupine bean in Australia (7). CMV has a global distribution and has the widest host range within plant viruses and infects a wide range of dicotyledonous and monocotyledon plants (8, 9). The virus causes different symptoms on various hosts, for example, mosaic, stunting, and yield-reducing of cucumber, leaf size reduction and fern leaf of tomato, chickpea leaf tip necrosis, soybean dwarfing, iris ring spot, chlorosis of banana, chlorosis, severe stunting, mosaic, malformation of young leaves and buds growth inhibition of spinach and so quantitative and qualitative yield reduction (10). The genome of the cucumber mosaic virus is multi-partite positive-sense single-stranded RNA (+)-ssRNA that is divided into three spherical particles with 28-30 nm diameter. Each RNA strand has a cap-like structure at the 5' end and a tRNA-like structure at the 3' end (10). In nature, CMV is transmitted by more than 80 species of aphids (11). A wide range of host of this virus is also a potential factor of its epidemics in important economic crops. The transmission efficiency of CMV varies from 1 to 50% in different plant species; CMV is seed-born in more than 20 plant species (1). Among the cultivated crops, seed transition is important in some species of *Cruciferae* and *Leguminosae* and with less important in *Cucurbitaceae* (13). CMV is more seed born in weed species, and this is very important in the virus epidemiology (14).CMV control is very difficult due to the wide host range and non-persistent transmission by more than 80 species of aphids. Hence, the best way to manage the disease caused by this virus is to cultivate resistant varieties (15, 16). *Cucumber Mosaic Virus* has already been reported in Iran from different hosts and areas (17). There was no information about the distribution, importance, and molecular characteristic of CMV in the cucurbitaceous fields of Jahrom. This study was done because it is important to continue basic research onto CMV importance, genetic diversity, and its host range in this area. This information leads to develop strategies for the management of disease and reduce its damage.

## Materials and Methods

*Samples Collection:* To identify and determine the spread of CMV from the main hosts of CMV, during the spring and summer of 2014, plant samples that were showing symptoms such as mosaic on leaf and fruit, mottle, blisters on fruits, fruit deformation, stunting, leaf deformation, and chlorosis were firstly collected from various farms of cucurbits and cucumber greenhouses of the city of Jahrom including Ghotbabad, Babaarab, Hydarabad, Chedrooye, Goldamcheh, Yousefabad, Simakan, Hormooj, Khafr and central part of Jahrom. The samples were from various cultivars of melon including Mashhadi, Talaei, Rina, Arna, Khatuni, and watermelon cultivars

including Charleston Gray, Niagara, Asguro and PS, and cucumber cultivars including American cucumber type, greenhouse cucumber cultivars including Negin and Sina, pumpkins including zucchini type, and then the samples were transferred to the virology laboratory. The total collected samples for investigation in the ELISA method were 147 samples including 26 cucumber samples, 35 pumpkin samples, 47 melon samples, and 39 watermelon samples. *ELISA Test:* The dispersion ratio of this virus was evaluated in mentioned regions using a direct double antibody sandwich (DAS) ELISA (Rocha-Pena et al., 1991). The polyclonal anti-serums used for the CMV was prepared from the Bioreba company of Switzerland. Extractions were prepared from 0.5 g of shoot and leaves in 5 ml of 1 × PBST buffer (0.15 M NaCl; 0.015 M NaH 2 $PO_4$; 0.05% Tween 20, pH 7.0). Positive reactions were defined as an OD 405nm two times higher than the negative control (18). *Nucleic acid extraction from tissues:* Total RNA (tRNA) was extracted from 0.2 g of shoot and leaves. First, tissues were pulverized in liquid nitrogen by pestle and mortar and then collected in a 1.5 ml sterile tube. Each sample was suspended in 400µl TES buffer (100 mM Tris-HCl pH 8.0; 2 mM EDTA; 2% w/v SDS) and 400 µl phenol/chloroform/ isopropanol (25/24/1) and shook vigorously for 10 min. After centrifugation (14000 rpm) for 10 min, the supernatant (400 µl) was treated with 200 µl ethanol (99.8%) and used in the RNeasy mini kit, and tRNA extracted according to the manufacturer's instructions and was used as a template for the amplification of the coat protein (CP) gene of CMV.

*Reverse Transcriptase (RT- PCR*): Primer pairs of $P_1$ (5′-TCCCAATGCTAGTAGAACCTCC-3′) and $P_2$ (5′-TGCTCGACGTCGACATGAAG -3′) were used to synthesize cDNA and amplify the coat protein gene of CMV. cDNA was synthesized using $P_2$ as a primer and total RNA extracted from plant samples. The final reaction volume was 20 µl. At first, 5 µl of the total RNA was mixed with 1µl $P_2$ primer and placed at 65° C in thermo-cycler for 5 minutes and then immediately was transmitted on ice. Subsequently, other reaction compounds, including 2µl of reverse transcriptase (RT) enzyme buffer, 1µl dNTPs (0.2mM each of the four dNTPs), and 0.5µl RT enzyme (Vivantis Company) were added. Contents were mixed gently and incubated at 42°C for 60 min and 85°C for 5 min, respectively. PCR amplification was performed in 20µl reaction mixture containing 13.5µl sterile distilled water (DDW), 0.5µl of each primer ($P_1$ and $P_2$), 5µl PCR master mix (Synagen Company), and 0.5µl cDNA. The PCR cycling profile was one cycle 95°C for 5 min, 30 cycles of 95°C for 50 s, 51° C for the 40s and 60s at 72°C and a final extension step for 15 min at 72°C. PCR amplified fragments were separated in 1% agarose gel in Tris-borate (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). After electrophoresis, gels were stained in 0.5 µg/ml ethidium bromide and observed and photographed by gel doc.*Sequencing and phylogenetic analysis of the CP gene of CMV:* Finally, two isolates from Ghotbabad and Hormoj regions were selected for the sequencing of the CP gene. The amplified products of approximately 618bp for the CP gene were sequenced. Multiple sequence alignment was performed using *Clustal W*, and a phylogenetic tree was constructed by *MEGA6* (Molecular evolutionary genetics analysis version 6.0) software (19), using the Neighbor-Joining method with 1000 bootstrap replications.

## Results

Results of the ELISA procedure showed that the highest percentage of CMV contamination was related to the melon (about 61%) and watermelon, pumpkin, and cucumber (46%, 20%, 19%), respectively. RT-PCR was performed using $P_1$ and $P_2$ primers on some of ELISA positive samples. The RT-PCR results also approved the CMV infection of these samples (Fig. 1). Nucleotide sequences of Two CMV isolates of melons of the Ghotbabad and Hormooj regions of the Jahrom area were performed. A comparison of these sequences with CP gene sequences of CMV in gene bank (NCBI) showed more than 98% nucleotide sequence homology. Multiple sequence alignment was performed using *Clustal W 1.6,* and a phylogenetic tree was constructed by *MEGA* software Version 6 (19), using the Neighbor-Joining(NJ) method with 1000 bootstrap replications (Fig. 2). Based on the phylogeny tree, these isolates have been located into two separate groups. Results showed a relationship between Jahrom isolates with some isolates of Australia, China, India, and South Africa and placed in one group but did not show any relationship with isolates of Japan, USA, and Malaysia.

## Discussion

In this study, the most common symptoms were observed in CMV positive samples were included mottling and blistering of leaves and fruits, but, shoestring was either very mild or did not exist. These symptoms were similar to symptoms reported by Zollanvari et al. (2012) (20). Among other symptoms induced by CMV on various cultivars of Cucurbits, in field and greenhouse condition of the Jahrom area, can account vein banding and leaf and fruit deformation. These symptoms were the same as Samiei et al. (2008) reports (21). In visiting the farms of Jahrom, CMV in cucumbers, melons, and pumpkin caused severe stunting, leaf deformation, and reduction of leaf size that were the same as symptoms reported by Sydanmetsa and Mbanzibwa (2016) (22).

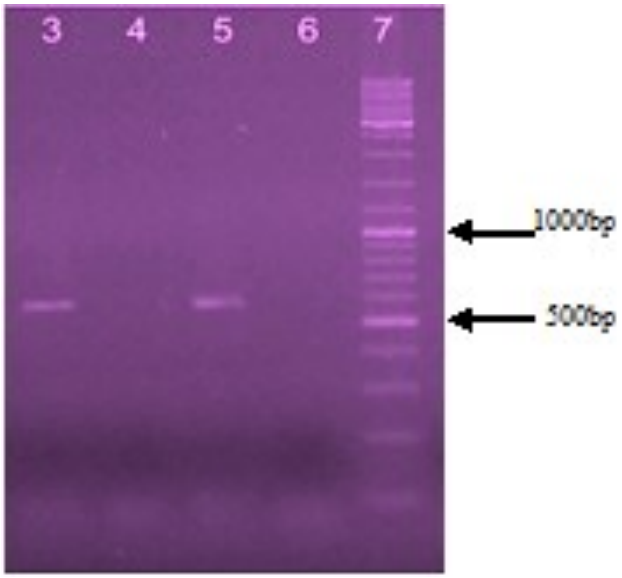


Fig. 1. Gel electrophoresis patterns obtained from RT-PCR. 3 and 5- positive samples (550 bp), 4- negative sample, 6- negative control, 7- Ladder 100-10000 (Fermentas). (Negative and positive samples based on ELISA results).

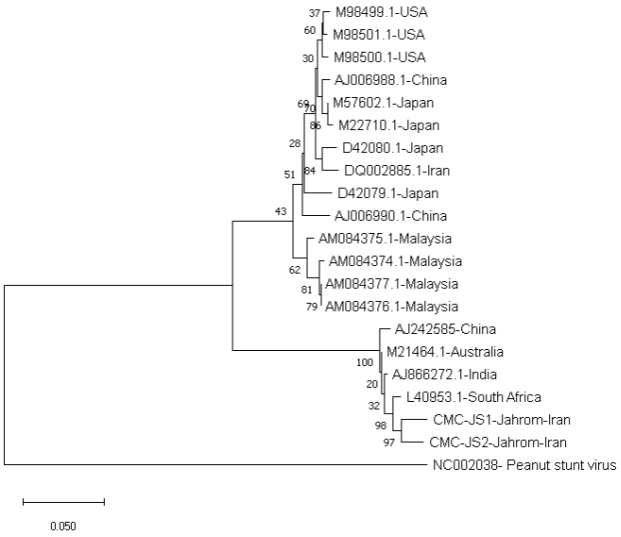


Fig. 2. The bootstrap (1000 replicates) phylogenetic tree of Cucumber Mosaic Virus isolates of Jahrom-Iran in comparison to 18 isolates from around the world. Peanut Stunt Virus is used as an outgroup.

The difference and variability of symptoms can be due to many factors such as mixed infection with other viruses, which may increase or decrease disease (23). One-hundred and forty-seven samples were collected from different cultivars of cucumber, melon, watermelon, and pumpkin from the Jahrom area, in which 59 samples showed CMV infection using the serological method. The highest percentage of CMV infection was related to melon (61%). Watermelon, pumpkin, and cucumber had 46%, 20%, and 19% infection, respectively. In another research, in the Jahrom area, the highest level of Watermelon mosaic infection was observed in melons with approximately 78% infection (24). These results are proving mixed infection of these viruses and are assenting with the description of Demeski and Sowell (1970) (25) that damage to cucurbit products by CMV is important. Using specific primers in the RT-PCR method, a DNA segment with a length of about 618bp was amplified. Phylogenetic analysis of the nucleotide sequence of the CP gene of two-isolates from the Jahrom area with sequences of the CP gene of 18 isolates of CMV from different regions of the world was performed using MEGA software and the Neighbor joining method. In comparison of CMV-JS1 (Accession number KU605635) and CMV-JS2 (Accession number KU605636) isolates of Jahrom area with isolates of worldwide, Jahrom isolates with isolates of Australia, China, India, and South Africa were located in one group. Phylogenetic tree analysis shows that an isolate with accession number DQ002885.1 that was from the North West of Iran is located far from Jahrom isolates. This distance shows differences between Iranian isolates.

## Conclusion

The results of this study showed that there where a significant infection in cucurbit fields of the Jahrom area and may cause significant yield losses. Therefore, it is necessary to investigate the incidence and mixed infections of this virus to estimate the rate of the virus damage ratio and to introduce resistant or tolerated varieties. Since the cucumber mosaic virus has a wide host range and is transmitted by aphids, hence it is recommended to control weeds as well as vector aphids. CMV infected plants attract aphids more than healthy plants, and this itself increases the rate of damage. Thus detection and elimination of diseased plants in fields and greenhouses reduce the damage significantly.

## Ethical Consideration

Authors of all ethics, including non-plagiarism, dual publishing has complied with data distortions and data making in this article.

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## Conflicts of Interest

The authors declare no conflicts of interest.

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