Akademik Ziraat Dergisi 12(2): 199-210 (2023)
ISSN: 2147-6403 e-ISSN: 2618-5881 DOI: http://dx.doi.org/10.29278/azd.1318370

Araştırma
(Research)

Biological and Molecular Detection of Cucumber mosaic virus (CMV) Isolates Obtained from Izmir

Sachin SHARMA[1*], Mustafa GÜMÜŞ[1]

[1]Ege University, Faculty of Agriculture, Department of Plant Protection, İzmir/TÜRKİYE

Alınış tarihi: 22 Haziran 2023, Kabul tarihi: 22 Kasım 2023
Sorumlu yazar: Sachin SHARMA, e-posta: sharmasachinneu@gmail.com

Abstract

Objective: This study was intended to determine cucumber mosaic virus (CMV) isolates present in tomato-growing areas of the Izmir province of Turkey in 2021-2022 based on mechanical inoculations and RT-PCR method.

Materials and Methods: 17 CMV suspected plant samples previously obtained from Izmir between 2019-2022 and preserved under the appropriate temperature in the Faculty of Agriculture, Department of Plant Protection of Ege University were used to carry out mechanical inoculation of the virus into a number of different test plants consisting of *Nicotiana glutinosa, Solanum lycopersicum* 'SC-2121', and *Cucumis sativus* 'Beit Alpha' cultivars. Virus-inoculated plants were visually evaluated for symptom development, followed by a DAS-ELISA test with CMV-specific antibodies. CMV-positive tobaccos were used to repeat mechanical inoculation of the virus into newly grown test plants, followed by a second ELISA and final RT-PCR test.

Results: As a result, CMV-D and CMV-B2 isolates obtained from Izmir produced CMV-like symptoms in the test plants. However, only CMV-D inoculated test plants consistently came out to be positive in the final ELISA and RT-PCR test. CMV-D isolate in tobacco and cucumber induced systemic mosaic and in tomato, caused mosaic, stunting and bushy appearance during 3 weeks of virus inoculation. CMV-D inoculated plants when tested for RT-PCR produced an amplified cDNA band of 280 bp in agarose gel indicating the presence of the virus.

Conclusion: This study concludes that CMV causes a variety of symptoms depending upon the viral strain involved, infected host species, and other factors. The presence of CMV-D isolate has been biologically and molecularly identified based on repeated mechanical inoculations in its host species and a final RT-PCR test performed under a controlled experimental setup. Further study of the responsible isolate can be achieved by its genome sequencing and phylogenetic analysis to better understand the viral strain involved in the infection.

Keywords: *Cucumber mosaic virus* (CMV), mechanical inoculation, DAS-ELISA, RT-PCR

İzmir'den Alınan Hıyar mozaik virüsü (CMV) İzolatlarının Biyolojik ve Moleküler Tespiti

Öz

Amaç: Bu çalışma, 2021-2022 yıllarında Türkiye'nin İzmir ilinin domates yetiştirilen bölgelerinde bulunan Hıyar mozaik virüs izolatlarının mekanik inokulasyon ve moleküler RT-PCR yöntemlerine dayalı olarak belirlemesini amaçlamışır.

Materyal ve Yöntem: Daha önce 2019-2022 yılları arasında İzmir'den temin edilen ve Ege Üniversitesi Ziraat Fakültesi Bitki Koruma Bülümü iklim odalarında uygun sıcaklıkta muhafaza edilen CMV izolatlarıyla enfekte şüphesi olan 17 bitki örneği, *Nicotiana glutinosa, Solanum lycopersicum* 'SC-2121', ve *Cucumis sativus* 'Beit Alpha' kültüvarından oluşan çok sayıda farklı test bitkisine virüsün mekanik inokulasyonu gerçekleştirmek için kullanılmış. Virüs inokule edilmiş bitkiler, simptom gelişimi açısından görsel olarak değerlendirilmiş, ardından CMV'ye özgün antiserumlarla DAS-ELISA testi yapılmıştır. CMV pozitif tütünler virüsün yeni yetiştirilen test bitkilerine mekanik inokulasyonu tekrarlamak için inokulum kaynağı olarak kullanılmış, ardından ikinci bir ELISA ve nihai RT-PCR testi yapılmıştır.

Araştırma Bulguları: Bulgularda, İzmir'den temin edilen CMV-D ve CMV-B2 izolatları test bitkilerinde CMV benzeri simptomlara neden olmuştur. Ancak, son ELISA ve RT-PCR testinde yalnızca CMV-D izolatı ile inokule edilen test bitkilerinin sürekli olarak pozitif olduğu ortaya çıkmıştır. CMV-D izolatı, virus inokulasyonundan 3 hafta sonra tütün ve hıyarda sistemik mozaik, ve domateste mozaik, bodurluk ve çalılaşma görünümüne neden olmuştur. CMV-D izolatı RT-PCR için test edildiğinde agaroz jelde virüsün varlığını gösteren 280 bp'lik cDNA bandı gözlenmiştir.

Sonuç: Bu çalışma, CMV'nin ilgili viral ırka, enfekte konukçu türlere ve diğer faktörlere bağlı olarak çeşitli semptomlara neden olduğu sonucuna varmaktadır. CMV-D izolatın varlığı, kontrollu bir deney düzeneğinde konakçı türde tekrarlanan mekanik inokulasyona ve son RT-PCR testine dayalı olarak biyolojik ve moleküler olarak tespit edilmiştir. Enfeksiyona neden olan viral ırkın genetik özellikerini daha iyi anlamak için gelecek çalışmalarda ilgili izolatın genom dizilimi ve filogenetik analizi ile daha fazla bilgi sağlanması amaçlanmaktadır.

Anahtar kelimeler: Hıyar mozaik virüs, mekanik inokulasyon, DAS-ELISA, RT-PCR

Introduction

Tomato (*Lycopersicon esculentum* L; *Solanaceae*) (Diez and Nuez, 2008) is a major vegetable crop with an important economic and socio-cultural value to the world. Tomato fruits are known to contain fiber, protein, carbohydrates, vitamin C, vitamin K, vitamin B9, Zn, Cu, S,e, Mn, Ca, Mg, K, Fe, Lycopene, Beta-carotene, Naringenin, Chlorogenic acids, antioxidants, and several other compounds that have important health benefits (USDA, 2021). Globally, Turkey is the third biggest tomato producer after China and India (FAO, 2021), and the Mediterranean, Aegean, and Marmara regions of Turkey together contribute nearly 68% of the total tomato production in the country (TÜİK, 2021). Tomato production and productivity in Turkey have been facing several challenges, one of them is the cases of frequent viral disease infections that pose a constant threat to the tomato industry. Cucumber mosaic virus (CMV) is one of the widespread plant viruses identified in different parts of Turkey based on several serological and molecular detection techniques (Değirmenci and Uzunoğulları, 2007; Uzunoğulları and Gümüş, 2015; Güneş and Gümüş, 2019). Cases of CMV infection in vegetable fields of Aegean province have been reported as well (Özkan, 1957; Özlap, 1964; Gümüş, 1998; Güneş et al., 2023). CMV can infect more than 1200 plant species belonging to 101 families and is one of the oldest plant viruses found throughout the world (Doolittle, 1916; Jacquemond, 2012). It causes disease in several vegetables including tomatoes, peppers, cucumbers, and so on. Depending on the viral strain involved in the infection the symptoms may vary from yellowing, mottling, or mosaic of leaves, leaf deformation, narrow leaves, bushy appearance, and stunting. One of the distinguishing symptoms of CMV infection in tomatoes is shoe-string in which the infected leaflets of the plant are fully suppressed (Palukaitis et al., 1992; Akhtar et al., 2008; Zitter and Murphy, 2009; Mahjabeen et al., 2012). Depending upon the diseased plant species and the involvement of other factors, CMV infection could have a multitude of effects on the infected plants such as; reduced plant growth, root length and weight, decreased fruit weight, yield, and variable effects on the production of phenolic compounds in infected tomatoes (Mahajabeen et al., 2012). Similarly, the virus is known to impair the photosynthetic abilities and respiration rates of infected tomato, cucumber, and melon plants (Song et al., 2009; Shalitin and Wolf, 2000). CMV can readily be transmitted by nearly 75 aphid species in a non-propagative and non-circulative manner (Palukaitis et al., 1992). In addition, the virus can be transmitted mechanically, through seeds of some plants and *Cuscuta* species (Palukaitis et al., 1992; Roossinck, 2001; Jacquemond, 2012). CMV is a positive sense RNA virus, icosahedral in shape, made up of three RNAs (RNA1, RNA2 and RNA3) and most of the CMV strains are differentiated into three subgroups (IA, IB and II) based on their serological and molecular specifications (Palukaitis et al., 1992; Roossinck, 2001; Çağlar, 2006; Karanfil et al., 2023). It is, therefore, crucial to correctly identify CMV strain and study its pathogenic nature, host range, and the variety of symptoms it causes in its hosts, which would aid in devising an appropriate management strategy, such as developing disease-resistant crop varieties in the future.

The present study was intended to characterize cucumber mosaic virus isolates obtained from tomato-growing regions of the Izmir province of Turkey based on virus inoculation in a range of different plant species and RT-PCR method. The objective of the study was to understand and

differentiate the types of symptoms induced by CMV isolates mainly in tomatoes and a few other plant species in a controlled experimental condition and to further verify the results based on molecular diagnosis.

Material and Method

Biological Detection

Plants suspected of CMV infection that had been acquired from tomato-growing regions of the Izmir province and preserved under an appropriate temperature (-80 °C or -20°C, before and during inoculation) in the Department of Plant Protection of Ege University were used to carry out mechanical inoculation of the virus into a number of test plants consisting of *Nicotiana glutinosa*, *Solanum lycopersicum* 'SC-2121' and *Cucumis sativus* 'Beit Alpha' cultivars. Virus-inoculated plants were visually evaluated for symptom development for 3 weeks, followed by a DAS-ELISA test with CMV-specific antibodies for further confirmation. ELISA-positive tobaccos were used to repeat the mechanical inoculation of the virus isolates into newly grown test plants made up of the same species, followed by a second ELISA and a final RT-PCR test.

Growing of test plants

At least two (or more) test plants made up of each species were used in mechanical inoculations for each isolate. From growing of test plants to inoculating them with CMV isolates and evaluating symptom development on them was performed in an experiment room with an average temperature of 24-24 °C, 40-41% Relative humidity and ample supply of light (16 hours of exposure) and water.

Mechanical Inoculation

17 plant samples (Table.1) were separately ground in mortar and pistils put under an ice-filled box. 5 ml, 0.05 M Potassium phosphate buffer having pH 7.2 and mixed with 1% Na_2SO_3 (Iqbal et al., 2011) was added to the slurry to avoid oxidation of virus particles and to facilitate their establishment into plant cells. The solution containing virus particles and buffer was filtered through a double-layered muslin cloth. Celite abrasive was sprinkled over the young leaves of test plants to create micro-wounds. The cotton swabs dipped in the filtered solution were lightly rubbed on the wounded leaves. Virus-inoculated plants were rinsed with tap water after 3-4 minutes and left for observation for 3 weeks. Cucumbers were virus-inoculated on their Cotyledon leaves whereas tomato and tobaccos were inoculated at their 3-4 leaf developmental stage.

Table 1. Cucumber mosaic virus (CMV) sample isolates used in mechanical inoculation

S.N.	Virus isolates	Plant samples	Sampling year
1.	CMV-7	Pepper	2019
2.	CMV-14	Pepper	2019
3.	CMV-29	Pepper	2019
4.	CMV-32	Pepper	2019
5.	CMV-37	Pepper	2019
6.	CMV-39	Pepper	2019
7.	CMV-62	Pepper	2019
8.	CMV-72	Pepper	2019
9.	CMV-73	Pepper	2019
10.	CMV-75	Pepper	2019
11.	CMV-77	Pepper	2019
12.	CMV-82	Pepper	2019
13.	CMV-K	Tobacco	2019
14.	CMV+TSWV-03	Tomato	2022
15.	CMV-B1	Pepper	2022
16.	CMV-B2	Pepper	2022
17.	CMV-D	Tomato	2022

Plants suspected or symptomatic of CMV infection after 3 weeks of post-inoculation were serologically tested for the presence of the virus using the DAS-ELISA procedure by following Clark and Adams (1977) guidelines and the manufacturer's protocol (Bioreba, AG Switzerland) with minor adjustments. The results of the ELISA plates were visually observed for the presence of CMV based on color formation in the sample-containing wells. Whereupon necessary the spectrophotometric absorbance value was measured with (Titertek multiscan plus MK II ELISA reader) at 405 nm wavelength and samples that had absorbance value equal to or more than 2 times the value of negative control were considered as positive.

Molecular Detection

Molecular detection consisted of Total Nucleic Acid (TNA) extraction of test plants, cDNA synthesis of TNA samples, RT-PCR amplification of cDNAs, and gel electrophoresis of PCR amplified products. Test plants symptomatic for the viral presence and/or those that came positive for CMV in the second DAS-ELISA test were included in the RT-PCR test. Asymptomatic and ELISA-negative test plants were excluded from PCR testing.

The Silica-based TNA extraction method outlined by Foissac et al., (2001) was followed with minor adjustments. A mixture of 30 mL Grinding buffer and 300 µL β-mercaptoethanol (MCE) was prepared and each sample consisting of 100 mg/leaves was ground in sterilized proselin bags by adding 2 ml of the prepared solution. 500 µL of the samples were transferred into separate eppendorf tubes labeled with sample information. Each tube was then loaded with 100 µL sarcosil (10%) and incubated at 70 °C in a block incubator for 10 minutes. Subsequently, the tubes were chilled on ice for 5 minutes and centrifuged at 14000 rpm for another 10 minutes. Meanwhile, new eppendorf tubes were labeled and supplied with 300 µL 6M sodium iodide (NaI) and 50 µL silica. 300 µL of centrifuged samples were then transferred into the new tubes containing sodium iodide and silica, later 150 µL ethanol was also added to each tube. The tubes were then briefly vortexed, shaken in a shaker for 10 minutes, and centrifuged at 6000 rpm for 1 minute. Afterward, the outer liquid portion of the samples was thrown out leaving tubes with nucleic acids attached to silica. The tubes were then washed with a mixture of 15 ml washing buffer and 15 ml ethanol solution. 500 µL mixture of washing buffer and ethanol was added to each tube and the tubes were vortexed until the silica inside was fully dissolved and then centrifuged at 6000 rpm for 1 min. The liquid portion of the tubes was thrown out, the washing procedure was repeated in the same manner and the liquid portion of the tubes was thrown out. Afterward, the tubes were adjusted on tissue paper in a slanting position and subjected to drying at room temperature for a few minutes. The tubes were then supplied with 150 µL RNA-free distill water, briefly vortexed, incubated at 70 °C for 4 minutes, and centrifuged at 14000 rpm for another 3 minutes. In the final step, the liquid portion of the samples was transferred into newly labeled eppendorf tubes. Each sample's total RNA concentration was measured using EzDrop 1000 Micro-Volume Spectrophotometer-Blue-Ray Biotech and the samples were preserved at -20 °C until further use. The TNA samples were converted to complementary DNAs by following the cDNA synthesis protocol provided by the manufacturer (Abm Onescript Plus cDNA Synthesis Kit) with minor adjustments. 5 µL of each TNA sample was mixed with 7.5 µL NFW, 1 µL dNTPs (10 mM), and 1 µL random primer (10 mM) forming a total volume of 9.5 µL for each. PCR microtubes filled with samples were then briefly centrifuged and heated at 65 °C for 5 minutes in an RT-PCR machine (Thermocycler). Afterward, 4 µL, RT buffer (5X), 1 µL RTase enzyme (200 U/µL), and 0.5 µL RNAse OFF Ribonuclease Inhibitor (40 U/µL) was mixed per tube, and each tube containing sample was supplied with the total volume of 5.5 µL. PCR tubes were then subjected to the process of cDNA synthesis in an RT-PCR machine with a specific command for about an hour (25 °C for 10 min, 50 °C for 50 min, and 85 °C for 5 minutes). cDNA synthesized samples were preserved at -20 °C until further use. cDNA synthesized samples were PCR amplified in an RT-PCR machine with the presence of a CMV-specific forward and reverse primer set (Forward: 5' ACTCTTAACCACCCAACCTT 3' and Reverse: 5' AACATAGCAGAGATGGCGG 3') previously stated by (Faggioli et al., 2005; Lumia et al., 2001). The manufacturer's protocol (Abm OneScript Plus Reverse Transcriptase) was followed for the RT-PCR amplification with minor adjustments. Firstly, 2 µL of each cDNA synthesized sample was mixed with 8.5 µl nuclease-free water, 12.5 µL PCR master mix, 1 µL forward, and 1 µL reverse primer forming the total volume of 25 µL for each in separate PCR tubes. Samples were then subjected to the process of denaturation, annealing, and extension in an RT-PCR machine with CMV-specific command. The denaturation was completed at 95 °C for 3 minutes in 1 cycle, annealing at 94 °C for 30 seconds, 55 °C for 45 seconds, and 72 °C for 45 seconds in 35 cycles and finally extension was completed at 72 °C for 7 minutes in 1 cycle. A few representative of PCR-positive samples were reverified by amplifying them in the presence of a new primer set obtained from (Giakountis et al., 2018).

After PCR amplification, the samples were loaded on (agarose) gel electrophoresis and subjected to run for about an hour. Later the gel with samples was removed, visualized under a UV-transilluminator, and photographed.

Results

Results of biological detection

The CMV isolates used in this study for mechanical inoculation were found to cause disease and produce symptoms in the test plants during 3 weeks of post-inoculation. However, symptom development was slightly varied depending on the virus isolates tested and the plant species used for their inoculation. Among 17 CMV-suspected samples used for the virus inoculation, only CMV-D and CMV-B2 isolates consistently produced symptoms in the test plants when the mechanical inoculation was repeated after successful results in the first trial. Both of these isolates were contained in tomato and pepper plants obtained from Izmir. The pepper and tomato samples had leaf curling, leaf deformation, and mosaic-like symptoms suggesting one of the possibilities of CMV infection. Tobacco plants inoculated with CMV-D and CMV-B2 isolates produced necrotic local lesions after 4 days on the virus-inoculated leaves. Previous literature suggests that CMV is known to induce systemic mosaic to vein yellowing in *N. glutinosa* depending upon the virus strain involved in infection (https://www.dpvweb.net/dpv/name-index). The necrotic local lesions on inoculated leaves of tobacco implied the possibility of mixed infection of tomato and pepper samples with more than one virus. Necrotic local lesions containing leaves of *N. glutinosa* were plunged out in order to facilitate the systemic establishment of CMV into emerging leaves. CMV-D inoculated *N. glutinosa* started to show signs of systemic infection in its newer leaves after 14 days of virus inoculation. A mosaic with yellowing of the above leaves was observed in *N. glutinosa* after 3 weeks of CMV-D infection. Additionally, *Nicotiana tabacum* cv Xanthi was inoculated with CMV-D isolate for further confirmation of the biological reaction in which the isolate induced distinct vein banding on newly emerging leaves (Figure 1). CMV-B2 inoculated tobaccos (*Nicotiana glutinosa*) did not produce any distinct symptom during 3 weeks of post-inoculation (Figure 2).

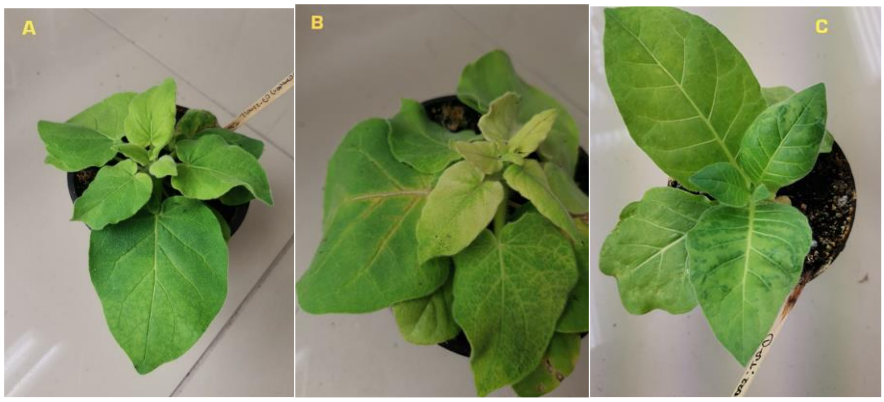

Figure 1. (A & B)- Nicotiana glutinosa 1 inoculated with CMV-D isolate is showing early signs of systemic infection after 14 days (A) and yellowing with mosaic symptoms after 21 days (B) respectively. (C) *N. tabacum* cv. Xanthi inoculated with CMV-D is showing distinct vein banding in its emerging leaves after 21 days of post inoculation

Figure 2. (D & E)- *N. glutinosa* inoculated with CMV-B2 isolate are asymptomatic after 21 days of post inoculation

CMV-D inoculated tomatoes (*Solanum lycopersicum* SC 2121) started to produce mosaic symptoms in their emerging leaves after 2 weeks of virus inoculation. The mosaic pattern was more pronounced after 3 weeks accompanied by a mild form of leaf deformation. One of them was stunted with reduced leaf size and a bushy appearance (Figure 3).

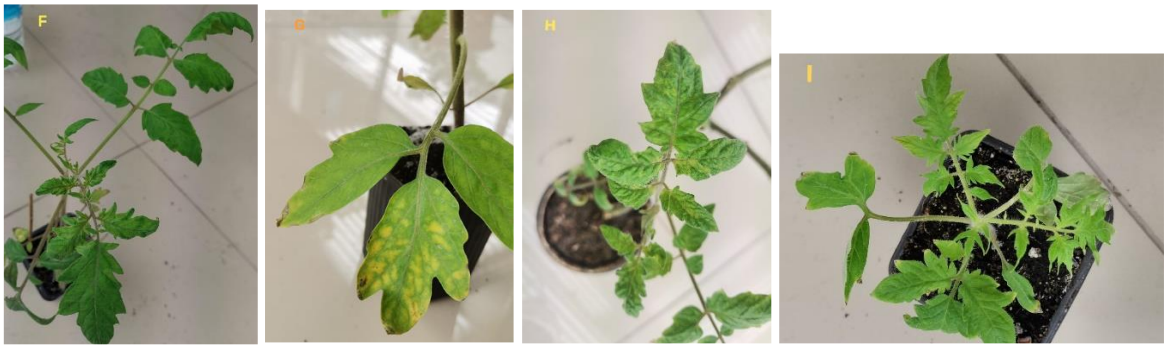

Figure 3. (F & G)- *Solanum lycopersicum* 1 inoculated with CMV-D is showing a mosaic of its younger and older leaves. (H & I)- *S. lycopersicum* 2 and 3 inoculated with CMV-D are showing mosaic, mild leaf deformation, and reduced leaf size with a bushy appearance respectively

CMV-B2 inoculated tomatoes (*S. lycopersicum* SC 2121) started to produce mosaic in their newer leaves after 2 weeks of virus inoculation. The mosaic pattern became more distinct after 3 weeks with lower leaves showing yellowing symptoms (Figure 4).

Figure 4. (J & K)- S. lycopersicum 4 and 5 inoculated with CMV-B2 are showing distinct mosaic and yellowing of lower leaves.

CMV-D inoculated cucumbers (*Cucumis sativus* Beit Alpha) produced mild mosaic at the end of 3 weeks of post-inoculation. CMV-B2 on the other hand did not produce any distinct symptoms on inoculated cucumbers (*C. sativus* Beit Alpha) and the plants appeared to be stunted at the end of 3 weeks of virus inoculation (Figures 5).

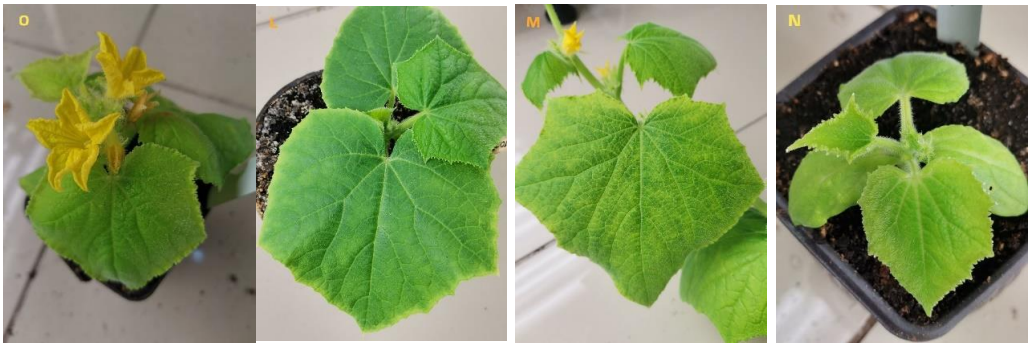

Figure 5. (L & M)- *Cucumis sativus* 1 and 2 inoculated with CMV-D are showing the mild form of mosaic. (N & O)- *C. sativus* 3 and 4 inoculated with CMV-B2 are stunted and asymptomatic

The DAS-ELISA test was conducted for the CMV-suspected or symptomatic test plants after 3 weeks of the first mechanical inoculation. As a result, CMV-D and CMV-B2 inoculated test plants came out to be positive for the presence of CMV. The ELISA positive tobaccos inoculated with these two isolates were used to repeat the mechanical inoculation of the virus isolates into newly grown test plants made up of the same species as before and symptom development was monitored for another 3 weeks. However, only CMV-D inoculated test plants gave CMV-positive results in the second DAS-ELISA test. CMV-B2 inoculated test plants, on the other hand, gave CMV-negative serological test results even though the virus-inoculated tomatoes were showing mosaic symptoms (Figure 6).

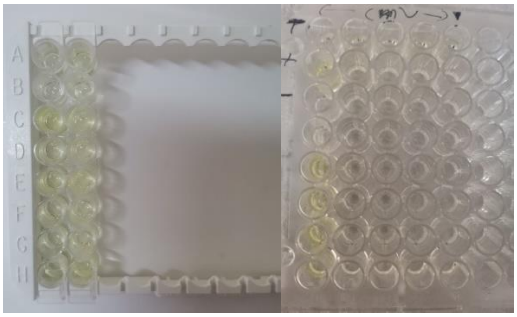

Figure 6. DAS-ELISA test results of CMV-D and CMV-B2 inoculated plants after first (left) and second (right) mechanical inoculations.

Test plants inoculated with the rest of the isolates either failed to produce any distinct symptom and/or failed to give CMV-positive Elisa results after the first mechanical inoculation and thus were removed from the experiment. For instance, three isolates namely CMV-39, CMV-75, and CMV-77 did not produce any distinct symptoms in the test plants after the first mechanical inoculation but they gave close to positive DAS-ELISA results for CMV taken afterward. However, when the mechanical inoculation and DAS-ELISA test were repeated, all of the test plants inoculated with these three isolates gave CMV negative results. Another sample with CMV+TSWV-03 isolate had been suspected of containing both *Cucumber mosaic virus* and *Tomato spotted wilt virus* (TSWV), when inoculated to the test plants induced necrosis of leaves in tobacco and tomato and no distinct symptom in cucumber during 3 weeks of the evaluation period. The infected plants when tested for the DAS-ELISA using specific antibodies of both CMV and TSWV gave positive results for TSWV but negative results for the presence of CMV and thus it was later removed from the experiment. The summary of biological detection is provided in (Table 2).

Table 2. Biological detection results of CMV inoculated test plants.

S.N.	CMV-suspected sample isolates	Viral symptoms on inoculated test plants			DAS-ELISA test results (first/last)	Explanations
		N. glutinosa	*S. lycopersicum*	*C. sativus*		
1.	CMV-7	**	**	**	--/	**+?** (Positive suspected) **++** (Positive) -- (Negative) ** (No symptom) **S** (Stunting) **SM** (Systemic Mosaic), **SN** (Systemic Necrosis), **LD** (Leaf Deformation)
2.	CMV-14	**	**	**	--/	
3.	CMV-29	**	**	**	--/	
4.	CMV-32	**	**	**	--/	
5.	CMV-37	**	**	**	--/	
6.	CMV-62	**	**	**	--/	
7.	CMV-72	**	**	**	--/	
8.	CMV-73	**	**	**	--/	
9.	CMV-82	**	**	**	--/	
10.	CMV-K	**	**	**	--/	
11.	CMV-B1	**	**	**	--/	
12.	CMV-39	**	**	**	+?/--	
13.	CMV-75	**	**	**	+?/--	
14.	CMV-77	**	**	**	+?/--	
15.	CMV+TSWV-03	SN	SN	**	--/	
16.	CMV-B2	**	SM	S	++/--	
17.	CMV-D	SM	SM, LD, S	SM	++/++	

Results of Molecular Detection

CMV-D inoculated test plants that were symptomatic and gave positive results for the presence of CMV in the second DAS-ELISA test and CMV-B2 inoculated tomatoes that were symptomatic for viral infection but gave CMV-negative Elisa results were included in molecular detection. The molecular detection consisted of Total Nucleic Acid (TNA) extraction, cDNA synthesis, RT-PCR amplification, and gel electrophoresis. The measurement of total RNA

concentration present in the TNA samples was performed using EzDrop 1000 Micro-volume Spectrophotometer Blue-Ray Biotech and the results suggested that test plants inoculated with both of these isolates had enough concentration of RNA in their system to suggest viral infection. However, when TNAs were converted to cDNA, RT-PCR amplified with CMV specific primer set and subjected to run in the gel electrophoresis, only CMV-D inoculated test plants gave positive PCR results by producing an expected nucleotide band of 280 bp in the agarose gel when visualized under UV transilluminator. In total, 5 PCR amplified test samples representing CMV-D isolate came out to be positive in the PCR test. PCR-amplified products of CMV-B2 inoculated tomato samples, on the other hand, failed to produce any bands in agarose gel and thus came out to be negative for the presence of CMV. The TNA samples used for PCR amplification were randomly chosen and examined in gel electrophoresis (Figure 7).

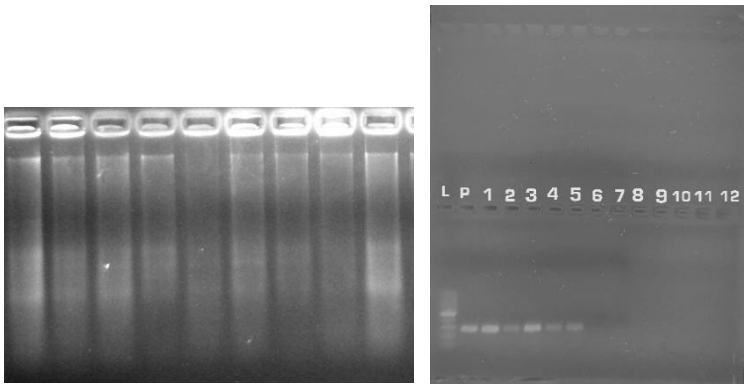

Figure 7. Gel electrophoresis results of TNA samples (left). RT-PCR test results of CMV-D and CMV-B2 inoculated plants (right); (L- 100 bp DNA ladder, P- Positive control, 1 to 12- PCR amplified samples. Positive test results have an approximate nucleotide band of 280 base pairs (bp)).

TNAs of previously CMV-positive PCR samples (CMV-D inoculated) were randomly selected for cross-checking by another PCR amplification using a new primer set F- GTTTTGTTTGTTGGAGAGTTGCG and R-GGGTCCTGTAGAGGAATGTGACATT) (Giakountis *et al*., 2018)) with an expected nucleotide band of 350 bp as a result. The nucleotide BLAST result of this primer set can be obtained with a Gene Bank ID of J02059.1 via the NCBI database.

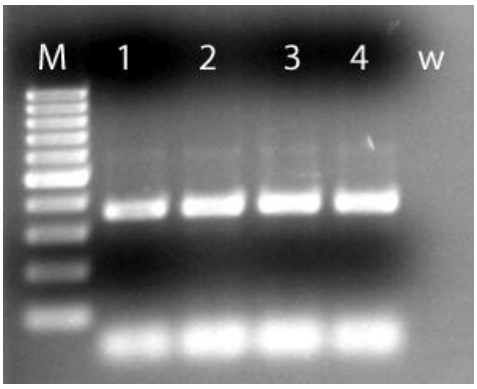

Figure 8. RT-PCR test results of cross-examined CMV-D inoculated test plants M- 100 bp DNA ladder, 1 to 4 PCR samples (Positive test results have nucleotide fragments of about 350 bp)

Discussion

A study was carried out to detect İzmir isolates of cucumber mosaic virus (CMV) based on mechanical inoculations and molecular RT-PCR method. Results of biological detection have revealed that among 17 CMV suspected plant samples that were mechanically inoculated to a number of different test plants consisting of *Nicotiana glutinosa*, *Solanum lycopersicum* 'SC-2121', and *Cucumis sativus* 'Beit Alpha', CMV-D and CMV-B2 isolates induced CMV-like symptoms on a consistent basis when the inoculation was repeated under the similar experimental settings. CMV-D in tobacco (*N. glutinosa*) and cucumber (*C. sativus* Beit Alpha) induced systemic mosaic and in tomato (*S. lycopersicum* SC 2121), caused mosaic, stunting, and bushy appearance during 3 weeks of the evaluation period. CMV-B2 induced mosaic and yellowing of leaves in tomato (*S. lycopersicum* SC 2121), stunting in cucumber (*C. sativus* Beit Alpha), and no distinct symptom in tobacco (*N. glutinosa*). The initial DAS-ELISA test for CMV after the first mechanical inoculation gave positive results for both CMV-D and CMV-B2 inoculated plants. However, only CMV-D inoculated test plants came out to be positive in the second DAS-ELISA test taken after the second

mechanical inoculation. In the same way, only CMV-D inoculated test plants gave CMV-positive RT-PCR results in the final. CMV-B2 inoculated test plants failing to give CMV-positive results in the final ELISA and PCR test could suggest that the viral isolate under study couldn't establish itself in the test plants after the second mechanical inoculation due to some unforeseen reason. An additional study of CMV-D isolate can be achieved by performing its genome sequencing and phylogenetic analysis to better understand the viral strain involved in infection, however, it is outside the scope of this work. The type and severity of symptom development induced by CMV in its host can vary depending on the sensitivity and age of the host plant, environmental factors such as temperature, and the viral strain involved in infection (Green and Kim, 1991; Palukaitis et al., 1992; Salánki et al., 2018). The molecular PCR test takes the genetic makeup of the pathogen under study into account and thus it is considered to be more sensitive than both biological assaying and serology-based ELISA test for the detection of plant viruses. Researchers have expressed the importance of using multiple methods for better detection and reliable results for plant viruses (Legrand, 2015; Hou et al., 2020). *N. glutinosa* has been extensively used as a propagative host for CMV in experimental settings where the viral strains have caused systemic mosaic to vein yellowing with varied severities. *Nicotiana glutinosa* and *N. tabacum* have been used for the propagation of CMV where the virus has induced mosaic symptoms (Değirmenci and Uzunoğulları, 2007; Regenmortel and Mahy, 2009; Mochizuki and Ohki, 2012). Tomato is one of the important hosts of CMV and depending upon viral strain and other factors involved symptom development varies from mosaic, mottling, leaf deformation, reduced leaf size, bushy appearance, stunting, shoe-string of leaflets and internal browning of fruits (Değirmenci and Uzunoğulları, 2007; Zitter and Murphy, 2009; Mahjabeen et al., 2012; Giakountis et al., 2018). CMV infection in cucumber could result in mosaic, mottling, vein clearing, vein banding, and leaf deformation among others (Değirmenci and Uzunoğulları, 2008; Plapung et al., 2014). Usually, CMV strains are known to induce mosaic and stunting in plants belonging to the families of *Solanaceae*, *Cucurbitaceae*, *Leguminosae*, *Brassicaceae,* and *Gramineae,* whereas in *Vigna unguiculata*, *Chenopodium amaranticolor* and *C. quinoa* the virus causes local necrotic lesions on the inoculated leaves (Mochizuki and Ohki, 2012).

Previously, cases of CMV infection from Istanbul and Izmir regions of Turkey in Pepper, Spinach, Celery (Özkan, 1957), and CMV infection in squash, and tomatoes grown in the Aegean region (Özlap, 1964) have been reported. CMV detection based on disease indexing in the indicator plants- N. tabacum, S. lycopersicum and C. sativus has been performed based on samples obtained from vegetable fields of the Aegean region in which the infected plants have exhibited a range of symptoms such as mosaic, yellowing, and leaf curling, narrowing of leaves among others (Özlap, 1964). Similarly, CMV has been detected in the infected peppers from the Aegean region based on serological and molecular tests (Gümüş, 1998; Güneş et al., 2023). In fact, CMV is one of the common plant viruses known to infect vegetable fields identified from different regions of Turkey based on ELISA and/or PCR tests. For instance, CMV infection in tomatoes has been reported in the eastern Mediterranean (Güllü and Çali, 1994), and Marmara regions as well (Değirmenci and Uzunoğlullari, 2007; karanfil and Korkmaz, 2021). Furthermore, the virus has been identified infecting gladiolus, squash, Cucumber (Uzunoğullari and Gümüş, 2015), tobacco (Usta et al., 2020), broccoli, onion (Sevik, 2017; Santosa and Ertunç, 2021), beans (Karanfıl and Korkmaz, 2017), lemon and grapevine (Paradies et al., 2000) and several other plants including cold seasonal crops (Sevik, 2019) from diverse parts of Turkey that briefly points out an extent of its dispersal and host range in the country. The phylogenetic analysis of the nucleotide and amino acid sequences of CMV isolates obtained from various regions of Turkey has indicated that the majority of isolates belonged to the CMV sub-groups IA and IB (Karanfıl et al., 2023; Güneş et al., 2023; Usta et al., 2020; Kurtoğlu and Korkmaz, 2018; Karanfıl and Korkmaz, 2017; Sarı, 2015). However, more studies based on the population dynamics of CMV isolates encompassing larger areas could further explore this matter. In this study, similar primer sets have been used (Faggioli et al., 2005; Giakountis et al., 2018) to amplify the virus particles leading to a positive result for the one isolate tested. The results of biological and molecular characterization of the CMV isolate used in this study align with the results of previous similar studies.

Conclusion

This study concludes that CMV causes a variety of symptoms depending upon the viral strain involved, infected host species, and other factors.

The presence of CMV-D isolate has been biologically and molecularly detected based on repeated mechanical inoculation in its host species and a final RT-PCR test performed in a controlled experimental setup. Further study of the responsible isolate can be achieved by its genome sequencing and phylogenetic analysis to better understand the viral strain involved in the infection.

Conflicts of Interest

The authors declare no conflicts of interest.

Authorship contribution statement

SS: Planning and conducting experiments, analyzing results and writing the manuscript.

MG: Planning and supervising experiments, reviewing results and written work.

Kaynaklar

Akhtar K. P., Ryu K. H., Saleem M. Y., Asghar M., Jamil F. F., Haq M. A., & Khan I. A. (2008). Occurrence of Cucumber mosaic virus Subgroup IA in tomato in Pakistan. *Journal of Plant Diseases and Protection*, *115*(1), 2–3. https://doi.org/10.1007/BF03356231

Çağlar, B.K., (2006). *Hıyar mozaik virüsü (CMV)'nün kavun (CMV-K), domates (CMV-D), biber (CMV-B) izolatlarının biyolojik, serolojik, moleküler yöntemlerle karakterizasyonu ve satellit RNA'lerin virüs üzerindeki etkisi*., Doktora Tezi, Çukurova Üniversitesi Fen Bilimleri Enstitüsü, 89 s.

Clark M. F., & Adams A.N. (1977). Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology, 34*, 475-483.

Değirmenci K., & Uzunoğulları, N. (2007). Determination of problem viruses on the tomatoes cultivation areas in Marmara region. *Bitki Koruma Bulteni*, *47* (1-4), 72-77. ISSN: 0406-3597.

Diez, M. J., & Nuez, F. **(**2008). *Tomato In: Prohens, J. and Nuez, F., Eds., Vegetables II. Springer, New York (NY)*, DOI: 10.1007/978-0-387-74110-9_7.

Doolittle, S.P. (1916). A new infectious mosaic disease of Cucumber. *Phytopath*, *06*, 145-147.

Faggioli, F., Ferretti, L., Albanese, G., Sciarroni, R., Pasquini, G., Lumia, V., & Barba, M. **(**2005). Distribution of olive tree viruses in Italy as revealed by one-step RT-PCR. *Journal of Plant Pathology*, 49-55 pp.

FAO (2021, September). *Food and Agriculture Organization of the United Nations. FAOSTAT*. Available from https://www.fao.org/faostat/en/#data.

Foissac, X., Svanella-Dumas, L., Dulucq, M. J., Candresse, T., Gentit, P. **(**2001). Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). *Acta Hortic*, *550*, 37–44. https://doi.org/10.17660/ActaHortic.2001.550.2

Giakountis, A., Tsarmpopoulos, I., & Chatzivassiliou, E. K. (2018). Cucumber mosaic virus Isolates from Greek Legumes are Associated with Satellite RNAs that are Necrogenic for Tomato. *Plant Disease*, *102*(11), 2268–2276. https://doi.org/10.1094/PDIS-08-17-1259-RE

Green, S. K., & Kim, J. S. (1991). Characteristics and control of viruses infecting peppers: A literature review. *Asian Vegetable Research and Development Center Technical Bulletin*, *18*, 60p.

Güllü, M., & Çali, S. (1994). Studies on the Determination and the Incidence of Virus Diseases of Protected Vegetable Crops in the East Mediterranean Region. Plant Protection Bulletin, 34(3), 79-93.

Gümüş, M. (1998). *İzmir İlinde biberlerdeki viral hastalık etmenlerinin ve oranlarının saptanması ve bazı biber çeşitlerinin bu virüslere reaksiyonlarının belirlenmesi*. (Yayınlanmamış Doktora Tezi). Ege Üniversitesi Fen Bilimleri Enstitüsü, Bitki Koruma Anabilim Dalı, 118s.

Güneş, N., & Gümüş, M. (2019). Detection and Characterization of Tomato spotted wilt virus and Cucumber mosaic virus on Pepper Growing Areas in Antalya. *Journal of Agricultural Sciences*, *25*(3), 259–271. https://doi.org/10.15832/ankutbd.499144

Güneş, N., Paylan, İ.C. & Gümüş, M. (2023). Molecular Characterization of Tomato Spotted wilt virus (TSWV) and Cucumber mosaic virus (CMV) Affecting Tomato and Pepper Crops in İzmir Province. *Ege Üniv. Ziraat Fak. Derg*. *60*(1), 67-79.

Hou, W., Li, S., & Massart, S. (2020). Is There a "Biological Desert" With the Discovery of New Plant Viruses? A Retrospective Analysis for New Fruit Tree Viruses. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.592816

Iqbal, S., Ashfaq, M., & Shah, H. **(**2011). Biological characterization of Pakistani isolates of Cucumber mosaic virus (CMV). *Pakistan Journal of Botany*, *43*(6).

Jacquemond, M., **(**2012). Cucumber Mosaic Virus. Advances in Virus Research, 84: 439–504. Elsevier. https://doi.org/10.1016/B978-0-12-394314-9.00013-0

Karanfil, A., & Korkmaz, S. (2017). Çanakkale ili börülce üretim alanlarında Hıyar mozaik virüsü (Cucumber mosaic virus; CMV)'nün tespiti ve kılıf protein genine göre moleküler karakterizasyonu. *Bitki Koruma Bülteni*. *57*(3), 293–304.

Karanfil, A. & Korkmaz, S. (2021). Güney Marmara bölgesi kabakgil üretim alanlarında Cucumber mosaic virus enfeksiyonunun tespiti ve kılıf protein gen diziliminin filogenetik analizi. *Ege Univ. Ziraat Fak. Derg*. *58*(2), 239-246. https://doi.org/10.20289/zfdergi.682293

Karanfil, A., Randa-Zelyüt, F. & Korkmaz, S. (2023). Prevalance of Cucumber mosaic virus (CMV) Isolates Infecting Tobacco Plants in Turkey and Population Structure of Turkish CMV Isolates. *Türk Tarım ve Doğa Bilimleri Dergisi*. *10*(3), 598-604.

Kurtoğlu, A. & Korkmaz, S. (2018). Determination and Molecular Characterization of Cucumber Mosaic Virus (CMV) Infection on Spinach Production Fields of Çanakkale Province, Turkey. *Journal of Turkish Phytopathology*, 47 (2), 43-51.

Legrand, P., **(**2015). Biological assays for plant viruses and other graft-transmissible pathogens diagnoses: A review. Bulletin OEPP/EPPO Bulletin, *45*(2). https://onlinelibrary.wiley.com/doi/abs/10.1111/epp.12222

Lumia, V., Ilardi, V., Tomassoli, L., & Barba, M. (2001). Transgene detection in industrially processed genetically modified tomato. *Petria*, *11*, 159-165.

Mahjabeen., Akhtar, K. P., Sarwar, N., Saleem, M. Y., Asghar, M., Iqbal, Q., & Jamil, F. F. **(**2012). Effect of cucumber mosaic virus infection on morphology, yield and phenolic contents of tomato. *Archives Of Phytopathology And Plant Protection*, *45*(7), 766–782. https://doi.org/10.1080/03235408.2011.595965

Mochizuki, T., & Ohki, S. T. (2012). Cucumber mosaic virus: Viral genes as virulence determinants. *Molecular plant pathology*, *13*(3), 217-225. DOI: 10.1111/J.1364-3703.2011.00749.X

Özkan, M., (1957). Türkiye Sebze Ziraatinde Hıyar Mozaik Virüsü. *Tomurcuk*, *6*(65), 16-21.

Özlap, M O., (1964). Izmır Ili Civarında Görulen Önemli Sebze Virüsleri Üzerinde İncelemeler. *Bitki Koruma Bülteni*, *4*(1), 18-25.

Palukaitis, P., Roossinck, M. J., Dietzgen, R. G., & Francki, R. I. B. **(**1992). Cucumber mosaic virus. *Advances in Virus Research*, *41*, 281-348.

Paradies, F., Sialer, M. F., Gallitelli, D., Castellano, M. A., Di Franco, A., Digiaro, M., Martelli, G. P. & Yilmaz, M. A. (2000). Partial Characterization of Cucumber Mosaic Virus Isolates From Citrus and Grapevine. *Journal of Plant Pathology. 82*(2), 133–145.

Plapung, P., Khumsukdee, S., & Smitamana, P. (2014). Development of Cucumber lines resistant to Cucumber mosaic virus by Ovule Culture. *Journal of Agriculture Technology*, *10*(3), 733-741.

Regenmortel, M. V., & Mahy, B. **(**2009). *Desk Encyclopedia of Plant and Fungal Virology*. Academic Press ELSEVIER. https://www.elsevier.com/books/desk-encyclopedia-of-plant-and-fungal-virology/van-regenmortel/978-0-12-375148-5

Roossinck, M. J. (2001). *Cucumber mosaic virus*, a model for RNA virus evolution. *Molecular Plant Pathology*, *2*(2), 59–63. https://doi.org/10.1046/j.1364-3703.2001.00058.x

Salánki, K., Gellért, Á., Nemes, K., Divéki, Z., & Balázs, E**. (**2018). Molecular Modeling for Better Understanding of Cucumo virus Pathology. *Advances in Virus Research, 102*, 59–88. Elsevier. https://doi.org/10.1016/bs.aivir.2018.06.002

Santosa, A., & Ertunc, F. (2021). Characterization of two Cucumber mosaic virus isolates infecting *Allium cepa* in Turkey. *Phytopathologia Mediterranea*, *60*(1), 13-21.

Sarı S. (2015). *Samsun ilinde yetiştirilen yazlık sebzelerde enfeksiyon oluşturan Cucumber mosaic virus (CMV) izolatlarının karakterizasyonu ve konukçu-simptom-satellit RNA ilişkilerinin araştırılması.,* Yüksek Lisans Tezi, Ondokuz Mayıs Üniversitesi Fen Bilimleri Enstitüsü, 89s.

Sevik, M. A. (2017). Natural occurrence of cucumber mosaic virus infecting broccoli in Turkey. *Virus disease*. *28*(2), 218–219. https://doi.org/10.1007/s13337-017-0373-9

Sevik, M. A. (2019). Viruses infecting cool season crops in the northern Turkey. *Anais Da Academia Brasileira De Ciencias*. *91*(3), e20180224. https://doi.org/10.1590/0001-3765201920180224

Shalitin, D., & Wolf, S. (2000). Cucumber Mosaic Virus Infection Affects Sugar Transport in Melon Plants. *Plant Physiology*, *123*(2), 597–604. https://doi.org/10.1104/pp.123.2.597

Song, X.S., Wang, Y.J., Mao, W.H., Shi, K., Zhou, Y.H., Nogués, S. & Yu, J.Q. (2009). Effects of cucumber mosaic virus infection on electron transport and antioxidant system in chloroplasts and mitochondria of cucumber and tomato leaves. *Physiologia Plantarum*, *135*(3), 246–257. https://doi.org/10.1111/j.1399-3054.2008.01189.x

TÜİK (2021, Nisan). *Türkiye İstatistik Kurumu. Tarim Ürünleri Piyasaları DOMATES Ocak 2021*. Erişim adresi https://tarimorman.gov.tr.

USDA (2021, April). *United States Department of Agriculture. Food Data Central Search Results: Tomato, Roma*. Available from https://www.usda.gov/.

Usta, M., Güller, A. & Günay, A. (2020). The molecular characterization of the coat protein sequence and differentiation of CMV- subgroup I on tobacco from native flora in Turkey. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca.* 48(2), 523–534. https://doi.org/10.15835/nbha48211822

Uzunoğullari, N., & Gümüş, M. (2015). Detection of Cucumber mosaic virus (CMV) causing natural infection in some culture plants in Marmara region. *Trakya University Journal of Natural Sciences*, *16*(1), 9-15.

Zitter T. A., & Murphy, J. F. (2009). Cucumber mosaic. *The plant health instructor*. DOI: 10.1094/PHI-I-2009-0518-01.