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Response of Some Pepper Genotypes to Cucumber Mosaic Virus (CMV) and Discrimination of Kilis Isolates Using High-Resolution Melting (HRM) Method

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ABSTRACT

The escalating global population, diminishing agricultural lands, and the overarching global climate crisis are significantly impacting pepper cultivation. These challenges exacerbate the vulnerability of pepper plants to various biotic and abiotic factors, particularly viral diseases, resulting in diminished yield and quality. Cucumber mosaic virus (CMV), a notable concern for peppers in the Solanaceae family, is causing substantial quality and yield losses, with no effective chemical control methods currently available. This study focuses on exploring the genetic structure of CMV isolates obtained from pepper production areas in Kilis province and comparing these regions through the High-Resolution Melting (HRM) method. CMV isolates, cultivated in tobacco plants, have their partial coat protein sequences compared with those of other CMV isolates registered in the gene bank. The nucleotide sequences of identified CMV isolates are phylogenetically grouped and compared using an HRM graph. The HRM graph effectively distinguishes Kilis 3 and Kilis 4 isolates, clustered similarly to sequence patterns, from other isolates. The study highlights the utility of HRM analyses in identifying differences between isolates before determining sequence patterns. In the gene bank comparison, Kilis CMV isolates distinguished from others. Similarities were observed with isolates from Iran's Balsam (*Impatiens balsamina* - LC066478), Türkiye's Radish (*Raphanus sativus* - LC0665051), and Wild Turnip (*Rapistrum rugosum* - LC066514, LC066511, LC066517). The study found that Kilis 7 CMV isolate, transferred mechanically to 24 different pepper genotypes (*C. annuum*) from the local population, revealed susceptibility to CMV in the 24 lines developed from the Elbeyli population in Kilis province.

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Introduction

Determination and conservation of agricultural biodiversity hold critical importance for the sustainability of agricultural production and plant diversity (Mijatović et al., 2013). Vegetables, which are intensively cultivated worldwide in almost every region, constitute essential nutrients and have a wide range of products (Ebert, 2020). Among these vegetables, pepper, a significant member of the Solanaceae family, is used not only for fresh consumption but also in pickles, paste, canned products, sauces, or frozen items (Hong and Gruda, 2020).

Pepper's country of origin is the Americas, and the first and most comprehensive taxonomic study on this plant was conducted by Eshbaugh (1970) and also by Hunziker (1979). These studies concluded that 25 species identified in the *Capsicum* genus were not economically significant. However, recent discoveries have revised this number to 43 (Mavi, 2020). Among these species, five (*C. annuum* L., *C. frutescens* L., *C. baccatum* L., *C. chinense* Jacq., and *C. pubescens* Ruiz & Pavon) have been domesticated, with *Capsicum annuum* being the most widely grown commercially (Wang and Bosland, 2006).

Pepper, which holds a significant place in vegetable cultivation in Türkiye, is grown in large areas in every region of the country. It is one of the important species processed for fresh consumption or in industrial vegetable farming, with considerable commercial potential. Peppers are commonly cultivated in Mexico, China, Türkiye, and many other places. According to the 2021 data presented by the Food and Agriculture Organization of the United Nations (FAO), the total production of various pepper species is around 36.29 million tons. China leads this production with 16.72 million tons, followed by Türkiye with 3.09 million tons and Indonesia with 2.75 million tons. Mexico and Spain rank fourth and fifth, respectively (FAO, 2021). A significant portion of pepper production in Türkiye, especially in Antalya, Mersin, and Hatay provinces, takes place under protected cultivation. Other important regions for open-field pepper production include Samsun,

Manisa, Bursa, Izmir, and Canakkale. Additionally, peppers produced in Şanlıurfa, Gaziantep, Kilis, and Kahramanmaraş are used extensively in the manufacturing of pepper powder and flakes (Güller et al., 2020).

The increase in global population, decrease in agricultural land, and the impact of the global climate crisis are severely affecting the agricultural sector (Bozoglu et al., 2019). This situation is exacerbating the influence of various biotic and abiotic factors on pepper cultivation. Virus diseases represent a significant biotic factor causing yield loss and a reduction in fruit quality in pepper plants. However, as in the case of other pathogens, there is no effective chemical method for controlling viral diseases (Parisi et al., 2020). The expansion of plant trade worldwide has increased the prevalence of viral agents and vectors. The inability to apply chemical control methods and reliance on resistant varieties and preventive measures make the fight against viral agents more challenging. Furthermore, changing environmental conditions further emphasize the importance of viral diseases (Nicaise, 2014).

In the last 30 years, there has been a substantial increase in the number and prevalence of virus species causing infections in pepper production areas in tropical and subtropical regions. The reasons for this increase include the growth of pepper production areas, the increasing density of pepper cultivation, the movement of products carrying viruses to new regions along with the growth in trade volume and speed, and climate change expanding the suitable geographical range for viruses and vectors (Martelli and Quacquarelli, 1982; Yoon et al., 1989; Florini and Zitter, 1987; Green and Kim, 1991).

Plant virus diseases pose a significant threat to pepper cultivation due to the lack of chemical control methods. These diseases disrupt the normal development pattern of plants, causing economic losses by preventing normal fruit formation. Virus diseases have special significance among living agents due to aspects such as their size, chemical and physical structure, type of infection, reproduction within the plant, transmission, symptom formation, and the absence of an effective control method (Yılmaz and Davis, 1985). The most important virus after tobacco mosaic virus in harmful virus diseases of cultivated plants is CMV. This virus is widely found worldwide with approximately 1200 plant hosts in 101 families. Like Potyviruses, CMV is transmitted in a non-persistent manner by aphids. At least 86 aphid species have been identified as CMV vectors in peppers, with two of the most effective and widespread being *Myzus persicae* and *Aphis gossypii* (Moury and Verdin, 2012). While more than 70 virus infections have been reported in peppers (Kenyon et al., 2014), it has been reported that about 20 virus species in 15 different taxonomic groups economically damage pepper cultivation in the Mediterranean basin (Moury and Verdin, 2012). The increasing prevalence of different virus species also increases the risk of simultaneous infection of two or more virus species on the same plant. Genetic recombination and the exchange of genome fragments between different virus types not only increase the aggressiveness of virus species and their transmissibility by vectors but also weaken host resistance (Uzunoğulları and Gümüş., 2015). Additionally, CMV is a virus that has a helper virus function for the replication of satellite RNAs. Satellite RNAs are small nucleic acid molecules that exist in nature and are unrelated to plants, dependent on a helper virus for their replication, and associated with the genome of a helper virus. They may show genetic similarity to the genome of the helper virus. In both cases, they need a helper virus to replicate, transmit within the plant, and cause infection. Besides affecting the severity of symptoms created by the virus in hosts and weakening the host's resistance, some satellite RNAs are also used as control materials through cross-protection (Montasser et al., 1998; Monti et al., 1999). In this study, genetic characterization and HRM analysis of CMV isolates collected from pepper production areas in Kilis province were investigated to understand the responses of pepper populations to the pathogen.

Materials and Methods

Materials

Plant Materials

CMV isolates were multiplied in *Nicotiana tabacum* cv. Samsun. Twenty-four lines of *Capsicum annuum* L. peppers collected from Elbeyli district of Kilis province between 2012 and 2019 were utilized as plant material (Figure 1).

Viral materials

The viral material of the study was composed of pepper leaf samples infected with CMV, collected from Kilis and Gaziantep provinces between June and September 2020. Isolates Kilis 2, Kilis 3, Kilis 4, Kilis 5, Kilis 7, Kilis 9, Kilis 10 were utilized as viral material.

DAS-ELISA (Double Antibody Sandwich-Enzyme Linked-Immunosorbent Assay) Antiserums

The polyclonal antisera utilized for CMV diagnosis through DAS-ELISA were obtained from the AGDIA company.

Primers Used for Determining CMV Isolate Population Structure

The population structure of CMV pepper isolates was investigated using the polymerase chain reaction (PCR) method with primer sequences prepared from the coat protein (CP) regions of the virus genome (Table 1).

Figure 1. Fruit images of the pepper lines used in the study

Table 1. Primer sequences amplifying KP regions of the CMV genome

Region	Primer sequence (5′ to 3′)
CMV CP F	AGG TTC AAT TCC TCT TRC TCC
CMV CP R	AAC GGG TTG TCC ATC CAG

Table 2. PCR reaction profile used for amplification of KP regions according to CMV genome organization

Genome	PCR programme
CMV KP	1 min at 95°C 50 s at 55°C *40 cycles 1min at 72°C 7 min at 72°C

Figure 2. Mechanical inoculation of tobacco plants

Method

Mechanical Inoculation

Leaf samples, weighing 1.0 g, were placed in a chamber and crushed with the addition of 4 ml of 2% (w/v) diethyldithiocarbamate (DIECA) in 0.03 M phosphate buffer (Na2HPO4 [pH 7.0] Supplementary-1). The crushed mixture, containing 0.09 g of activated charcoal and carborundum, was applied to tobacco and pepper plants (Figure 2). Three minutes after inoculation, the leaf surface was rinsed with tap water (Moury et al., 2004).

DAS-ELISA Test

The diagnosis of CMV was conducted using the DAS-ELISA method with polyclonal antisera (AGDIA, France) (Clark and Adams, 1977). ELISA plates were coated with 150 µl per well of antiserum diluted at a ratio of 1:200 in coating buffer. The plates were kept at 37°C for 3 hours and 30 minutes. Afterward, the plates were washed three times with wash buffer for 3 minutes, followed by drying on paper towels. Leaf samples, weighing 1 g, were placed in a chamber and crushed with the addition of 4 ml of 2% (m/v) diethyldithiocarbamate (DIECA) in 0.03 M, pH 7.0 phosphate buffer (Na_2HPO_4). Plant sap was added to the plate wells in a volume of 150 µl. The plates were kept at +4°C for 16 hours. After washing the plates three times with wash buffer and dried by tapping on paper towels. Enzyme-labeled virus antisera, diluted at a ratio of 1:200 in conjugate buffer, were added to the plate wells in a volume of 150 µl. The plates were kept at 37°C for 3 hours and 30 minutes. Afterward, the plates were washed three times with phosphate wash buffer and then dried by tapping on paper towels. A substrate buffer was prepared with 1 mg ml^{-1} p-nitrophenylphosphatase, and 150 µl was added to the plate wells. After a 60-minute incubation at room temperature, the absorbance values of the plates were recorded at a wavelength of 405 nm using a spectrophotometer (Thermo Multiscan GO, USA).

Viral RNA Isolation

Viral RNA was isolated using Tri Reagent (Sela et al., 2012). Leaf and fruit samples (500 mg) were crushed in 2 ml phosphate (grinding) buffer using a porcelain mortar and pestle and stored at +4°C until use. Crushed samples were transferred to tubes, and 500 µl Tri Reagent was added, vortexed, and left at room temperature for 10 minutes. Subsequently, 200 µl chloroform was added to the samples, mixed thoroughly, and centrifuged at 14,000 rpm for 15 minutes. The clear liquid portion, remaining on top of the tube, was transferred to new tubes, making approximately 200-300 µl. Volume of 300 µl isopropanol was added and mixed in tubes were left at room temperature for 10 minutes. After centrifugation at 14,000 rpm for 15 minutes, the liquid portion was discarded, and the pellet was rinsed by1 ml of 70% ethanol. The tubes were gently vortexed, and centrifugated at 14,000 rpm for 5 minutes, the liquid portion was discarded, and tubes were dried. Pellets were solved in 50 µl of RNAse free water, and stored at -20°C.

Complementary DNA Synthesis and Polymerase Chain Reaction (PCR)

For the reverse transcription 2 µl RNA, 1 µl reverse primer and 11.7 H_2O were incubated in 70°C for 3 minutes and kept at +4°C for 5 minutes. The mixture including 4 µl 5X first strand buffer, 0.8 µl 10 mM dNTP and 0.5 µl 200 U μl^{-1} Maloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) enzyme (Vivantis, Malaysia). The synthesis reaction was carried out at 42°C for 1 hour and terminated by incubating at 70°C for 10 minutes.

PCR was performed using 2 µl cDNA in a 20 µl mixture. The mixture contained 18 µl of PCR reaction mix (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 200 µM of each dNTP, 120 pM virus-specific homologous and heterologous primers, 1U Taq DNA polymerase (Fermentas), and sterile water. PCR conditions are provided in Table 2. The DNA products obtained in the PCR amplicons were sequenced by MEDSANTEK A.Ş (Istanbul).

Genetic Structure of CMV-Pepper Isolates

The nucleotide sequence analysis of PCR DNA was conducted using heterologous primer sequences (MedsanTek, Ankara). Alignment was performed using ClustalW (Thompson et al., 1994) in MEGA-X program (Kumar et al., 2018). Nucleotide and amino acid similarity ratios with other isolates recorded in the genetic bank were compared using the Blastn program at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted using the Neighbor-joining method (Saitou and Nei, 1987) and Kimura 2-parameter model, with a bootstrap value of 1000.

Results and Discussion

Presence of CMV in Pepper Samples

The presence of CMV in samples collected from pepper production areas was determined using the DAS-ELISA test. The absorbance values of the samples at a wavelength of 405 nm on the spectrophotometer are presented in Table 3.

Table 3. ELISA results of samples collected from pepper production areas (From A1 to F8: samples, F8: negative control, and 9th column buffer solution)

	1	2	3	4	5	6	7	8	9
A	0.136	0.1632	0.1093	0.1027	0.1071	0.2727	0.3572	0.181	0.0598
B	0.1291	0.1688	0.1264	0.3579	0.1408	0.409	0.1247	0.3126	0.0618
C	0.3038	0.1095	0.1208	0.2807	0.4049	0.1706	0.2807	0.1084	0.0512
D	0.0999	0.1024	0.134	0.1368	0.1554	0.1574	0.4451	0.3235	0.054
E	0.1023	0.3789	0.3614	0.1221	0.6814	0.1421	0.1568	0.3151	0.0535
F	0.1236	0.4228	0.1218	0.1148	0.087	0.3527	0.3363	0.0989	0.0468

Figure 3. CMV symptoms observed on plants and fruits in pepper production areas

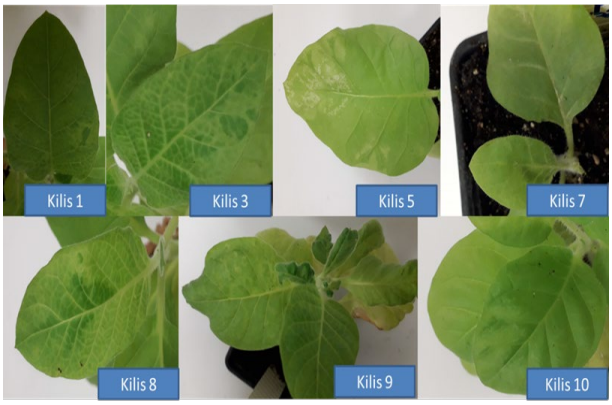

Figure 4. Symptoms of tobacco plants inoculated with Kilis CMV isolates

The ELISA plate included the negative control in the F8 well, with the 9th column filled with buffer. Samples showing an $O.D._{405}$ value more than three times the negative control (0.0989) were considered positive. Out of the collected 54 samples, 18 tested positive. Continuing research on the identification and characterization of the virus for the control of CMV outbreaks in pepper production areas will contribute to effective disease control and the discovery of new sources of CMV resistance. However, managing the widespread occurrence of CMV resistance, the partial resistance exhibited by resistance sources, the presence of many host ranges and vectors, and the potential seed transmission make disease control challenging.

In this study, the Kilis 7 CMV isolate was mechanically transmitted to 24 selected pepper genotypes (*C. annuum*) from the local population. The DAS-ELISA test results revealed that all selected local genotypes were susceptible to the pathogen. In tobacco leaves, symptoms such as blistering in the leaves, color changes between leaf veins, and darkening of leaf color in blistered areas were observed in Kilis 1, Kilis 3, and Kilis 8 isolates. Kilis 5 isolate exhibited color changes between leaf veins at leaf tips, while Kilis 7, Kilis 9, and Kilis 10 isolates showed deformities in leaf shape (Figure 3).

Response of Pepper Genotypes to CMV Isolate

In areas cultivating peppers, symptoms such as leaf discoloration, curling, mosaic patterns, elongation, and filiformity were observed (Figure 4). Fruit-related symptoms included depressions, color changes, chlorotic and necrotic areas, shrinkage in fruits, and the manifestation of leathery structures through the merging of necrotic areas in mature fruits.

Capsicum spp., commonly known as peppers, holds significant global importance as a vegetable and spice due to its color, flavor, and aroma (Rohini and Lakshmanan, 2017). Türkiye, with its pepper production for fresh consumption and drying, plays a crucial role in the Mediterranean basin. CMV is prevalent in pepper-producing regions and causes substantial damage among the known 70 viral diseases affecting pepper plants in countries cultivating peppers (Doolittle, 1916; Jagger, 1916; Palukaitis et al., 1992). Wild genotypes such as Pen 3-4 (C. baccatum), Vania, Gadir, and Nicklow's Emerald Bell (Pochard and Daubèze, 1989; Caranta et al., 2002), Perennial (C. frutescens) (Pochard, 1977; Nono-Womdim et al., 1993; Lapidot et al., 1997), and others have been reported to exhibit varying levels of resistance to the virus (Grube et al., 2000; Suzuki et al., 2003; Kang et al., 2010; Yao, 2013; Li and He, 2000; Wang et al., 2001; Mao et al., 2004; Sun et al., 2008).

Phylogenetic and HRM Analysis of CMV Isolates, and Responses of Local Genotypes to CMV

Upon comparison in the genetic database, Kilis CMV isolates were independently grouped. Isolate LC066478 from Balsam impatiens in Iran and isolate LC0665051 from Radish (*Raphanus sativus*) in Türkiye, along with isolates LC066514, LC066511, and LC066517 from Wild mustard (*Rapistrum rugosum*), were found to be similar (Ohshima et al., 2016). While Kilis CMV isolates formed two distinct groups among themselves, they were separated from other isolates (Figure 5).

For rapid identification of viruses, determination of mutations, and species differentiation, High-Resolution Melting (HRM) analysis is employed. This method has proven to be sensitive and fast enough to distinguish species with high accuracy. The analysis of HRM melt curves has been found to be sufficient to identify and distinguish species (Antonios et al., 2016).

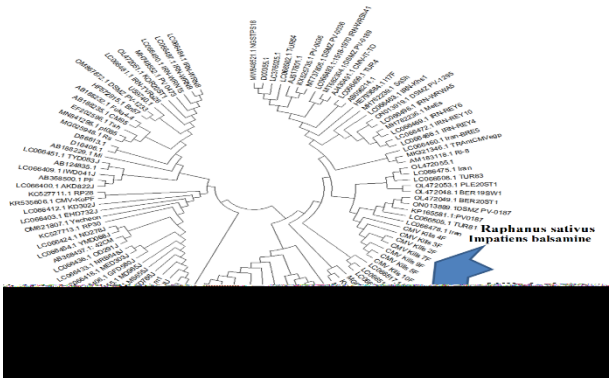

Figure 5. Comparison of Kilis CMV isolates with NCBI records

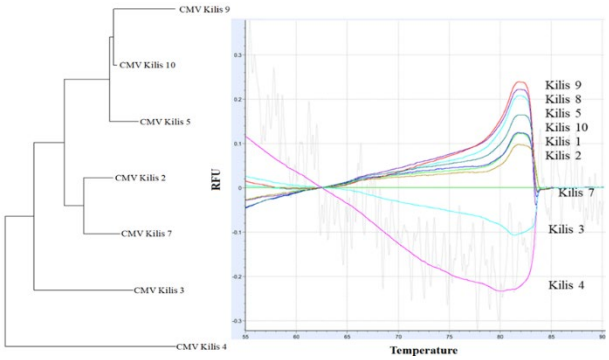

Figure 6. Phylogenetic analysis of CMV Kilis isolates (left) HRM discrimination graph of isolates (right)

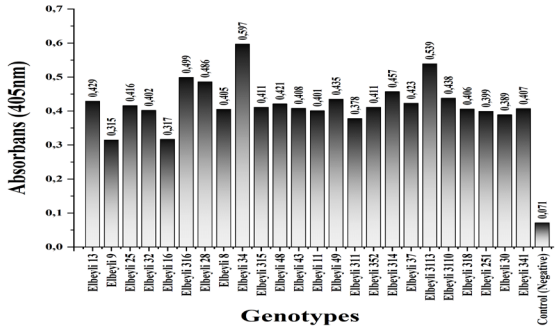

Figure 7. Absorbance values at 405 nm of Elbeyli genotypes infected with Kilis 7 CMV isolates, resistant lines and negative control

In this study, CMV isolates with determined nucleotide sequences were phylogenetically grouped and compared with an HRM graph containing these isolates. As a result of the comparison, Kilis 3 and Kilis 4 isolates, grouped similarly to sequence data, were separated from other isolates (Figure 6). The study indicated that HRM analyses could be used to determine differences between isolates before identifying sequence data.

Rapid screening of viruses is crucial for effective disease management. High-Resolution Melting (HRM) analysis is a rapid and highly accurate method for detecting genetic variations in DNA (Papavasileiou et al., 2016). This method allows the differentiation of species at the single nucleotide level (Ganopoulos et al., 2012). In our study, HRM analysis was effectively used in isolating different isolates, and the HRM graph showed results similar to phylogenetic analysis. The analysis of HRM melt curves has been suggested to distinguish Phytophthora species using the Ypt1 marker (Antonios et al., 2016). Similarly, HRM analysis with primers designed for GLRaV-3 virus in grapevines enabled the determination of different variants (I, II, III, and IV) (Bester et al., 2012).

Genotypes collected from Kilis province's Elbeyli district between 2012 and 2019 were inoculated with the Kilis-7 CMV isolate, and leaf samples were subjected to the DAS-ELISA test 14 days after inoculation. Readings at 405 nanometers were taken on the spectrophotometer 60 minutes after incubation with alkaline phosphatase, and the reading values are provided in Figure 7. All genotypes reflected values above the threshold, three times the negative control, and were identified as susceptible to the pathogen.

Conclusion

Considering the economic importance of pepper plants and their global production figures, the impact of CMV on these plants is a significant cause for concern. This study, by determining the responses of pepper populations in Kilis province to CMV, revealed high susceptibility of local genotypes to virus infection. The genetic characterization of CMV isolates collected from pepper production areas in Kilis province and the discrimination using High-Resolution Melting (HRM) analysis have been effective in identifying genetic differences between isolates. Kilis 3 and Kilis 4 isolates were successfully distinguished from others. The HRM method has proven to provide rapid and successful results in the diagnosis of viral diseases. The study is expected to contribute to the development of more effective strategies in combating virus diseases in pepper cultivation.

Declarations

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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