Investigation of Viruses Infecting *Lycopersicum esculentum* in Iran and Molecular Analysis of Cucumber Mosaic Virus

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Received: 2024-01-25 Accepted: 2024-03-18

Abstract

Viral diseases cause significant economic losses in tomatoes worldwide. This detect conducted a comprehensive survey in tomato (*Lycopersicum esculentum* L.) fields in Hamedan and Tehran provinces in Iran to detect and determine the incidence of tomato-infecting viruses. Using specific antibodies, collected symptomatic samples (348) were analyzed by Double antibody sandwich (DAS)-ELISA. According to the DAS-ELISA experiment, we found that 26.14% of collected samples were infected Arabis mosaic virus (ArMV), 36.78 % with Cucumber mosaic virus (CMV), 10.63% with Potato virus Y (PVY) 3.44 % with Tomato bushy stunt virus (TBSV), 7.18 % with Tomato spotted wilt virus (TSWV), and 2.87% with Tomato yellow leaf curl virus (TYLCV). Furthermore, double and triple infections were also observed in 15.08 and 6.03% of samples, respectively. CMV was the most prevalent among other tested viruses. Moreover, our findings showed that CMV was present in multiple infections of different samples. Serological diagnoses were confirmed by reverse transcription-polymerase chain reaction tests (RT-PCR) using a pair of primers that are specific for the detection of CMV and resulted in a DNA fragment of the expected size (540 bp). These results confirmed the DAS-ELISA experiment. Furthermore, in this study, we introduced a rapid method that facilitates the diagnosis of CMV in infected samples. Our findings can be used for control strategies and rapid diagnosis of viral infection in plants. Moreover, the outcome of this research can be used for the preparation of resistant cultivars against important viruses in tomatoes.

Keywords: CMV, DAS-ELISA, RT-PCR, Tomato, Viral diseases

Introduction

Infection to viral diseases has caused significant economic losses in tomato (*Lycopersicon esculentum*). Approximately more than 25 viruses from tomato were isolated globally (García-Estrada et al., 2022; Hanson 2022; Osundare, et al., 2023). Iran is a major producer of tomatoes, ranking seventh in the world according to the Food and Agriculture Organization (FAO) with a total production of 3,392,153.48 tons in 2021. Tomatoes are cultivated on over five million hectares worldwide. Because of the nutritional value of tomatoes, the

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cultivation of this crop is increasing year by year especially in the Middle-East and Mediterranean regions (García-Estrada, et al., 2022; Hanson, 2022; Rivarez et al., 2021). The tomato was domesticated by indigenous peoples in Latin America, due to its high nutritional value, has become a significant vegetable crop globally (Hanson, 2022). viral infections pose a serious threat totomato plants, with viral diseases being the primary factor responsible for reducing tomato yield (García-Estrada, et al., 2022; Hanson, 2022; Osundare, et al., 2023; Scholthof, et al., 2011; Zhang et al., 2022). Among the top ten plant viruses that infect plants six can affect tomatoes, including Tobacco mosaic virus, Tomato spotted wilt virus, Tomato yellow leaf curl virus, Cucumber mosaic virus, Potato virus Y, Cauliflower mosaic virus, African cassava mosaic virus, Plum pox virus, Brome mosaic virus, and Potato virus X (Scholthof, et al., 2011). Recent studies showed that viral diseases are the major limiting factor in the world's in tomato production worldwide, leading to significant losses (García-Estrada et al., 2022; Hanson 2022; Safaeizadeh et al., 2015). Most viruses that infect tomatoes exhibit symptoms such as mosaic, stunting, leaf distortion, leaf discoloration, dwarfing, spots, discoloration, and abnormality on fruit. The transmission of primarily occurs through aphids, insect vectors, most especially arthropods, and mechanical means (Hanson 2022; Osundare et al., 2023; Scholthof et al., 2011).

Hamedan and Tehran provinces situated in different agroecological conditions in Iran, are the main vegetable-growing-areas and there are ample opportunities for increasing tomato production in these areas.

Recently, virus diseases have been causing issues for tomato production in Iran (Masumi et al., 2009; Safaeizadeh, et al., 2015). The symptoms of these diseases can vary depending on the host, environmental conditions, and individual virus infection. However, some of commonly observed symptoms include leaf mosaics, mottling, crinkling, vein clearing, bushy growth, leaf rolling, leaf and plant shrinkage, vein purpling, shoe-string, chlorosis and necrosis, reduction in fruit size, and abnormal fruit color and shape, and plant death.

To date, numerous viruses infecting tomato plants have been reported from Iran, including Alfalfa mosaic virus (AMV; Alipour al., 2021), Arabis mosaic virus (ArMV; Massumi, et al., 2009), Beet curly top virus (BCTV; Kiumarsi and Karimie Rozbahani, 1995), Cucumber mosaic virus (CMV; Saidi, A., Safaeizadeh, 2012), Eggplant mottle dwarf virus (EMDV; Babaie and Izadpanah, 2003), Potato chlorotic stunt virus (PCSV; Danesh et al., 1989), *Potato virus Y* (PVY; Massumi, et al., 2009), Tomato bushy stunt virus (TBSV; Massumi, et al., 2009), Tomato mosaic virus (ToMV; Aghamohammadi, et al., 2013), Tomato spotted wilt virus (TSWV; Abadkhah et al., 2018), Tomato vein yellowing virus (TVYV; Ghorbani, 1993), and Tomato yellow leaf curl virus (TYLCV; Shirazi, et al., 2014). A destructive virus known as Tomato brown rugose fruit virus (ToBRFV) has recently been identified in the greenhouse tomato growing region of Isfahan province, Iran (Ghorbani, et al., 2021). ToBRFV

severely affect tomato production of the main greenhouse growing cultivars in Isfahan (Ghorbani, et al., 2021). Therefore, conducting a thorough investigation of the viruses infecting tomatoes can help in evaluating strategies against viruses. ToBRFV is previously reported by Salem et al., (2016) in Jordan, who classified it as a tobamovirus (Salem, et al., 2016). Given the significant economic losses caused by viral infections in tomatoes, particularly in light of high prices of the tomatoes in Iran in recent years, it is essential to identify the viruses associated with tomato yield losses and to study the occurrence of different viruses, whether as single or mixed infections in tomato fields. Molecular analysis of the most frequently found virus and the determination of phylogenetic status of several isolates, are crucial steps in elucidating virus epidemiology and controlling of plant viral diseases to introduce the appropriate varieties of tomato to farmers.

Material and methods

Sample Collection

A total of 348 leaf samples were collected from tomato fields in nine regions of Hamedan and eight regions of Tehran province. Each sample was labeled based on its geographical origin (Table 1). Symptoms of the diseases were recorded and the samples were stored in a portable fridge at 4°C before being sent to the laboratory for testing.

Serological studies and DAS-ELISA experiment

Tomato viruses were detected using highly specific polyclonal antibodies obtained from Bioreba (CH-4153 Reinach BL1, Switzerland) through double-antibody sandwich enzyme-linked immunosorbent assay (DAS)-ELISA following the protocol by Clark and Adams (1977) and manufacturer's instruction. All buffers, reagents, and positive and negative controls were obtained commercially sourced from Bioreba. Samples were ground in a sterile mortar and pestle with extraction buffer (PBST: 0.13 M NaCl, 0.014 M KH_2PO_4, 0.08 M Na_2HPO_4, 0.002 M KCl, pH: 7.4) containing 0.05% Tween 20 and 0.1% nonfat dry milk. The extract was added to the wells of the ELISA plate (Nunc Microwell, Roskilde, Denmark) pre-coated with IgG of ArMV, CMV, PVY, TBSV, TSWV, and TYCLV diluted in carbonate buffer (pH: 9.6). Following an incubation at 4°C the plates were washed four times with PBST-Tween 20 buffer (pH: 7.4). Subsequently, the plates were coated with alkaline phosphatase conjugated antibody diluted in the extraction buffer and incubated for 5 h at 30 °C. After washing, p-nitrophenyl phosphate in diethanolamine substrate buffer (0.5 µg/mL, pH: 9.8) was added to each well and incubated in dark condition at room temperature (18-25°C) for 30 to 120 min. The absorbance was measured at 405 nm using a microplate reader (ELx 800; Bio-Tek Instruments, USA). ELISA values were considered positive when the absorbance at 405 nm (A_{405}) was at least three times higher than the average of the negative controls (Massumi et al., 2009).

In order to confirm the identity of the DAS-ELISA results, sap taken from the positive samples for ArMV, CMV, PVY, TBSV, and

TSWV were inoculated in their propagative hosts, including; *Nicotiana clevelandii* Rose ex Vasey & Rose*, Nicotiana tabacum* cv. Samsun*, Datura stramonium* L., and *N. rustica* L.*,* respectively. The mechanical inoculation tests involved using leaf sap of the positive samples at DAS-ELISA prepared by macerating in 0.1 M potassium phosphate buffer, pH 7.2 (supplemented with 0.01 M ethylenediaminetetraacetic acid and 0.1% sodium sulfite) at a ratio of 1:10 (w/v) using a sterile mortar and pestle. The extract was then rubbed on carborundum-dusted test plants, with at least three plants used for each positive sample. Subsequently, the inoculated plants were maintained in an insect-proof greenhouse under standard conditions (15-25°C). Three weeks post-inoculation, the presence of the virus in both inoculated and uninoculated upper leaves of the test plants was assessed by DAS-ELISA. The inoculated test plants were kept in an insect-proof greenhouse and observed for 6 weeks for symptom development.

RNA extraction and reverse transcription-polymerase chain reaction tests

For molecular investigation and evaluation of CMV in positive samples using DAS-ELISA, reverse transcription-polymerase chain reaction tests (RT-PCR) were used. A total of 150 mg of the symptomatic areas of the leaves were used to extract RNA, following the protocol for manufacturers' protocol supernatant extraction technique with TRI-Reagent (Sigma, Chemical, St Louis, MO, USA) according to the extraction technique. Finally, the total RNA was re-suspended in 100 µl diethylpirocarbonate (DEPC)-treated H_2O. As the control, healthy tomato extracts were used. RNA quality and concentration were determined by gel electrophoresis through 1% agarose gels and nanodrop. The highly specific oligonucleotide primers, as detailed by Safaeizadeh et al. (2015); were used to amplify the conserved sequences of CMV RNA 3 resulting in540 bp fragment. The reverse primer (5-GCGCGAAACAAGCTTCTTATC -3) corresponds to nucleotides 633 to 653 in the non-coding intergenic region, while the forward primer (5-GTAATACGACTCACTATAGGTTTTGTT TG-3) is complementary to position 114 to 132 in the coat protein gene. The specific primers utilized in this study were synthesized by CinaClone. Co. based in Tehran, Iran. The cDNAs were generated with RevertAid^{TM}M-MuLV reverse transcriptase (M-MuLV, Fermentas, Germany), at 42°C for one hour, following the manufacturer's instructions with the addition of 0.5 µl RNAse inhibitor. Subsequently, 2.5 µl of the RT reactions were used for PCR in a total volume of 25 µl. The PCR procedure was performed using a thermal Cycler (T100 Bio-Rad, USA), starting with an initial denaturation step of 95°C for 5 min followed by 35 cycles of 60 s at 95°C, 60 s at 42°C annealing temperature (AT) and 60 s at 72°C. A final step of 8 min at 72°C ended the cycle. The resulting products and DNA ladder (GeneRuler, DNA Ladder Plus, CinaClone, Co. Tehran, Iran) were analyzed by electrophoresis on 1% agarose gels in the presence of 1 µg mL^{-1} ethidium bromide using 1 X Tris-Borate EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA, pH: 8.3; Green and Sambrook, 2014).

Results and Discussion

According to the DAS-ELISA data, it was observed that samples collected from Hamedan and Tehran provinces were infected with ArMV, CMV, PVY, TBSV, TSWV, and TYLCV. CMV was the most widespread virus infecting 36.78% of the total samples tested followed by ArMV (26.14%), PVY (10.63%), TSWV (7.18%), TBSV (3.44%), and TYLCV (2.87%). Double infections were detected in 14.01% and 15.08%, of samples obtained from Hamedan, and Tehran provinces, respectively (Table 1, and Figure 1). While triple infections were found in 3.18%, and 8.9% in Hamedan and Tehran provinces, in the same order (Table 1 and Figure 1).

In the current study, CMV was identified as the most prevalent viruses, affecting 35.81% and 39.26% of the samples collected from Hamedan and Tehran provinces, respectively. Notably, CMV infection was more widespread in the Bahar area of Hamedan province, where tomatoes are widely cultivated. In Tehran province, CMV infection was more common in Varamin region, known for its significant tomato

Table 1. Detailed distribution and occurrence of viruses collected samples in Hamedan and Tehran provinces

Province	Region	No. of samples Collected and tested	ArMV	CMV	PVY	TBSV	TSWV	TYLV	Double infections	Triple infections
Hamedan	Asad abad	22	2	9	1	0	1	0	3	0
	Bahar	28	2	13	7	0	0	2	4	2
	Lalehjin	10	0	5	1	0	0	0	1	0
	Famenin	24	5	8	1	2	0	0	3	0
	Razan	16	0	7	3	0	0	0	5	1
	Malayer	18	2	2	3	0	6	0	2	0
	Maryanaj	14	3	1	1	0	3	0	1	1
	Nahavand	18	11	6	4	2	0	1	1	1
	Tooyserkan	7	1	2	0	1	0	0	2	0
	Sum	157	26	53	21	5	10	3	22	4
	Percent of infection	--	16.56	35.81	13.37	3.18	6.36	1.91	14.01	3.18
Tehran	Karaj	35	11	13	5	0	0	2	7	2
	Varamin	42	12	16	7	5	6	3	5	4
	Robat karim	15	7	9	0	0	0	0	4	2
	Shahriar	23	12	12	1	1	1	1	6	3
	Mardabad	18	5	6	2	0	0	1	3	2
	Pakdasht	22	8	8	0	1	4	0	3	2
	Rey	19	4	5	1	0	2	0	2	1
	Eslamshhar	17	6	6	0	0	2	0	3	1
	Sum	191	65	75	16	7	15	7	33	17
	Percent of infection	--	34.03	39.26	8.37	3.66	7.85	3.66	17.27	8.9
	Total	348	91	128	37	12	25	10	55	21
	Percent of infection in two provinces	--	26.14	36.78	10.63	3.44	7.18	2.87	15.08	6.03

production. The data presented in Table 1 and Figure 1, indicated a higher incidence of ArMV in Tehran province compared to in Hamedan province.

According to Belval et al., (2021) that previously reported field's infection to *Xiphinema index* as the vector of nepoviruses could be one of the most important reasons of infection to ArMV in the studied regions. PVY is found to be prevalent in Hamedan province at 13.37%, while in Tehran province, the prevalence was 8.37%. These result could be attributed to the fact that Hamedan province's status as a major potato cultivation area in Iran, serving as a potential source of PYV transmission to other host plants. Plants with triple infections showed more severe leaf and fruit symptoms in this study compared to those infected with just one or two viruses. Moreover, a notable

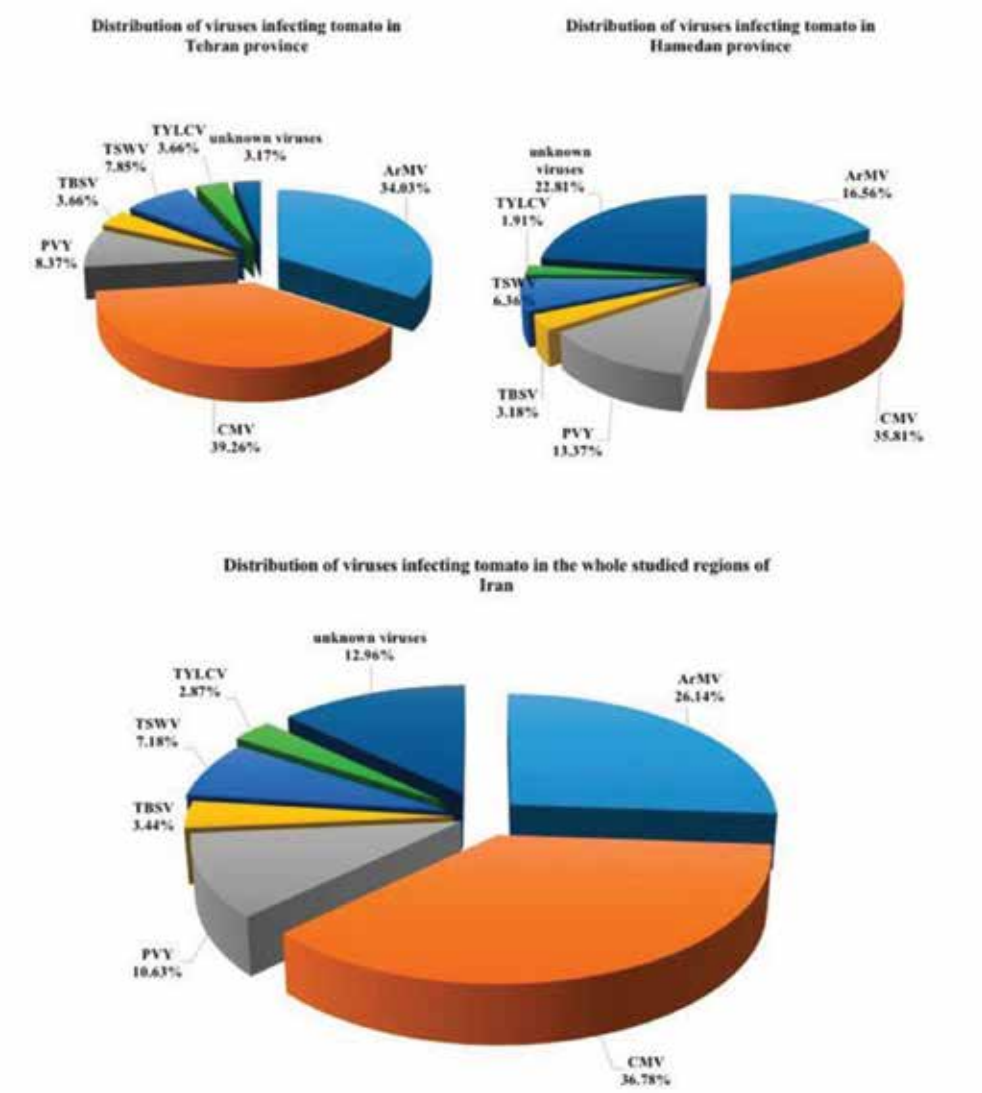

Fig. 1. Distribution of viruses infecting tomato (Lycopersicum esculentum) in Tehran and Hamedan provinces of Iran. Abbreviation: Arabis mosaic virus (ArMV), Cucumber mosaic virus (CMV), Potato virus Y (PVY), Tomato bushy stunt virus (TBSV), Tomato spotted wilt virus (TSWV), and Tomato yellow leaf curl virus (TYLCV)

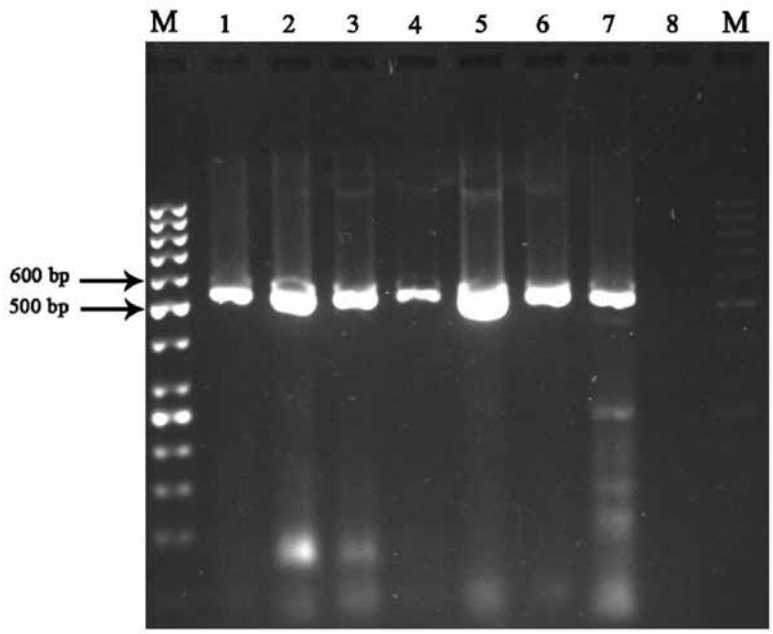

Fig. 2. Agarose gel of RT-PCR products for specific detection of Cucumber mosaic virus (CMV) infected tomato. M: molecular weight marker (DNA ladder). Numbers 1 to 8 indicate individual plants for each region corresponding as the confirmatory test to a positive sample in DAS-ELISA; three samples from Hamedan province (Bahar, Famenin, and Malayer; line number 1-3) and four samples from Tehran province (Karaj, Varamin, Mardabad and Pakdasht; line number 4-7). Line number 8: control (total RNA extracted from healthy *Lycopersicum esculentum*)

percentage of symptomatic samples did not show reactivity to any of the antibodies tested in this study, with rates of 22.89% and 3.17% observed in Hamedan and Tehran provinces, respectively.

The RT-PCR experiment was conducted as a confirmatory test for the positive samples in the DAS-ELISA analysis. Several samples from the studied area were tested, including three samples from Hamedan province (Bahar, Famenin, and Malayer) and four samples from Tehran province (Karaj, Varamin, Mardabad, and Pakdasht (Figure 2). The results of the RT-PCR analyses, using specific primers to detect CMV, were consistent with DAS-ELISA results. A PCR product of expected size (540 bp) was yielded from the infected samples, while no amplicon was generated from healthy plant extracts (Figure 2).

The presence of CMV was successfully identified using the specific primers (Figure 2). This finding is in line with the DAS-ELISA results confirming the accuracy of the DAS-ELISA experiment. The RT-PCR analysis produced a 540bp product, consistent with our evaluations. Therefore, this approach proves to be effective for prompt diagnosis of CMV in infected samples.

The results of the current study suggest that CMV strains affecting tomatoes in the surveyed area exhibited higher virulence levels and were in complete agreement with the elevated prevalence of CMV in those regions, shedding light on why CMV stands as the most widespread virus. CMV,

which leads to substantial economic losses in tomato crops, is primarily transmitted by aphids in a non-persistent manner on a global scale (Arinaitwe et al., 2022; Atarashi et al., 2020).

One crucial aspect that warrants further investigation in future research is the presence of symptomatic samples in the studied regions, that did not exhibit reactivity with any of the antibodies utilized in this study. The prevalence of such samples was 22.81% and 3.17% in Hamedan and Tehran provinces, respectively. These samples may potentially harbor other viruses such as Pepino mosaic virus (PepMV), Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), or even viroids that capable of infecting tomatoes (Anastassiadou et al., 2021; Choi, et al., 2020; Rivarez et al., 2021). A significant discovery is that the number of unidentified viruses in Hamedan province exceeded the number in Tehran province. It could be hypothesized that other unidentified viruses have been introduced from neighboring countries like Iraq and Turkey to the Hamedan province, given its proximity of these countries compared to the Tehran province. The unknown samples could include Tomato brown rugose fruit virus (ToBRFV) an emerging an RNA virus that is rapidly spreading (Zhang et al., 2022). This virus has been documented in numerous countries globally, and controlling its spread is crucial for tomato production (Zhang et al., 2022). Recent reports, have confirmed the prescence of ToBRFV in Turkey (Fidan et al. 2019); Ghorbani et al. (2021) in greenhouse complexes in Iran. Furthermore, Abou Kubaa et al., (2022), have reported this destructive virus from the Mediterranean region. Therefore, a thorough investigation into the identification of these unknown viruses is essential to develop an effective depth to formulate an adequate resistance program strategy.

In another research conducted in southeast and central regions of Iran to determine the prevalence of viruses infecting tomatoes, Massumi et al., (2009) identified ArMV as the most common viruses at 25.6%. They also found that CMV was the second most frequent virus affecting 23.4% of the studied samples. Although there are some discrepancies between their findings and our own research, this could be attributed to the significant differences in climate conditions between Hamedan and Tehran provinces (characterized by cold winters) and the southeastern and central regions of Iran, which can have a substantial impact on virus epidemiology. While TBSV has been previously reported in pelargonium and tomatoes from the Varamin region (Farzadfar et al. 2000). In the research conducted by Massumi et al., 2009 could TBSV did not detect in tomatoes, whereas we detected TBSV in 3.44% of collected samples.

In our study, numerous symptomatic samples did not exhibit reactivity towards any of the studied viruses. They may have been infected with alternative tomato-infecting viruses such as Tomato mosaic virus and Beet curly top virus previously reported by Massumi et al., (2009) with 4.8% and 6.1%, respectively. Also, they might be infected with other tomato-infecting viruses such as Pepino mosaic virus (PepMV) one of the

most important viral pathogens of tomatoes recently reported from other countries (Cho et al., 2022; He et al., 2020; Song et al., 2017). Moreover, they may be infected with Tomato leaf curl Palampur virus, a member of begomoviruses, with a rapid mutation rate and frequent recombination events that are a significant threat to tomato production worldwide and recently isolated from other hosts from Iran, neighboring countries, and many countries worldwide (Cai et al., 2023; Cao et al., 2024; Heydarnejad et al., 2009; Naganur 2023; Nayaka et al., 2024).

It is worth noting that the prevalence of CMV is attributed to its ability to infect various hosts including crop plants, vegetables, ornamental plants, and even weeds. Recently Safaeizadeh (2021), identified *Dianthus hybridus* as a new host for CMV in Hamedan province. In this study, the presence of CMV was confirmed using DAS-ELISA and RT-PCR techniques (Figures 1 and 2). Additionally, *Ibicella lutea* was previously reported as a new weed host for CMV (Safaeizadeh and Saidi, 2012). The prevalence of CMV in ornamental plants highlights the need for control strategies in greenhouses. Furthermore, Saidi and Safaeizadeh (2012) reported a severe strain of CMV from *Canna indica* that was cultivated in greenhouses in the Varamin region of Tehran province. These findings should be taken into consideration when developing strategies to control CMV in tomatoes. It is advisable to conduct a mechanical transfer of the study on these viruses to the model genetic plant A. thaliana in order to assess the symptoms in this plant. Subsequently, it is essential to analyze the hormonal changes in comparison to the wild type A. thaliana (Safaeizadeh and Ghotbi-ravandi, 2023).

The data presented in this study will be valuable in formulating future control strategies against viruses affecting tomatoes. However, the extent of crop and yield losses still needs to be determined and other properties of the studied viruses must be more studied. A comprehensive survey is also needed to investigate the occurrence of these viruses in other tomato fields across Iran and to evaluate the damage they are causing in terms of yield.

Acknowledgments

The authors express their gratitude to the researchers at the Iranian Research Institute of Plant Protection (IRIPP), specifically the Department of Plant Virology for their great help and advice during this research. Additionally, the authors wish to thank the Shahid Beheshti university research fund for their contribution to this project (research number: S/600/1153-99.10.21 and S/600/121-99.03.25). The authors also extend their thanks to their colleagues at the department of Cellular and Molecular Biology.

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