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Co-infection of *tomato brown rugose fruit virus* and *cucumber mosaic virus* in tomato in Iran

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Abstract

During surveys conducted in several greenhouses in northwestern and southwestern Iran in the fall of 2021-2022, viral symptoms such as leaf mosaic, leaf deformation, and shoestring were observed in several tomato plants. Reverse transcription polymerase chain reaction (RT-PCR) was performed using specific primers to detect the *tomato brown rugose fruit virus* (ToBRFV). A 458 base pair fragment corresponding to a part of the coat protein gene was amplified in all symptomatic samples. Sequence blast analysis and multiple alignment of the sequenced isolate with those of the isolates obtained from GenBank revealed 99.76% nucleotide sequence identity with existing GenBank isolates. Phylogenetic analysis grouped ToBRFV isolates into three clades, the Iranian isolate (Jol-F-2022) fell into clade II. The possibility of mixed infection in the samples with important viruses was investigated using universal orthotospovirus primers, *pepper mild mottle virus* (PMMoV), *tomato mosaic virus* (ToMV), and *cucumber mosaic virus* (CMV)-specific primers. In five samples, CMV-specific primers amplified a 657-base pair fragment of the envelope protein gene, whereas no fragment was amplified with general primers for the orthotospovirus genus and specific primers for PMMoV, ToMV. Nucleotide blast (BLASTn) analysis revealed a nucleotide sequence identity around 95.13-98.85 between the Iranian CMV isolate and the corresponding sequences in the GenBank. Phylogenetic analysis based on the nucleotide sequences of the coat protein gene grouped the CMV isolates into three major groups, the Iranian isolate grouped with the Indian CMV isolates in the subgroup IB. Biological studies were performed by mechanical inoculation of an infected sample on *Nicotiana rustica*. The inoculated plants showed severe mosaic symptoms at 15 days post inoculation (dpi), and infection by both viruses was confirmed through RT-PCR using specific primers. This study presents the first report of mixed infection of tomato plants with ToBRFV and CMV in Iran.

Keywords: Phylogentic analysis, Tomato, Emerging threat, Subgroup IB, Mixed infection.

آلودگی همزمان گوجه فرنگی با ویروس چروکیدگی قهوه‌ای میوه گوجه فرنگی و ویروس موزائیک خیار در ایران

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چکیده

طی بررسی‌های انجام شده در چندین گلخانه شمال غرب و جنوب غرب ایران در پاییز ۱۴۰۰-۱۴۰۱، علائم ویروسی از جمله موزاییک برگ، تغییر شکل و بدشکلی برگ و بند کفشی در چندین گیاه گوجه فرنگی مشاهده شد. واکنش زنجیره‌ای پلی‌مراز رونویسی معکوس (RT-PCR) با استفاده از آغازگرهای اختصاصی برای تشخیص ویروس چروکیدگی قهوه‌ای میوه گوجه فرنگی (ToBRFV) انجام شد. یک قطعه ۴۵۸ جفت بازی مربوط به بخشی از ژن پروتئین پوششی در تمام نمونه‌های علائم‌دار تکثیر شد. تجزیه و تحلیل بلاست توالی و هم ترازی چندگانه جدایه توالی‌یابی شده با جدایه‌های به‌دست‌آمده از بانک ژن، شباهت توالی نوکلئوتیدی ۹۹,۷۶ درصد را با جدایه‌های بانک ژن نشان داد. آنالیز فیلوژنتیک جدایه‌های ToBRFV را در سه کلاد گروه‌بندی کرد، جدایه ایرانی (Jol-F-2022) در کلاد دو قرار گرفت. احتمال آلودگی مخلوط نمونه‌ها با ویروس‌های مهم با استفاده از آغازگرهای عمومی ارتوتوسپوویروس‌ها (orthotospovirus)، و آغازگرهای اختصاصی ویروس پیسک خفیف فلفل (PMMoV)، ویروس موزائیک گوجه‌فرنگی (ToMV) و ویروس موزاییک خیار (CMV) مورد بررسی قرار گرفت. در پنج نمونه، آغازگرهای اختصاصی CMV یک قطعه ۶۵۷ جفت بازی از ژن پروتئین پوششی را تکثیر کردند، در حالی که هیچ قطعه‌ای با آغازگرهای عمومی برای جنس ارتوتوسپوویروس و آغازگرهای اختصاصی برای PMMoV وToMV تکثیر نشد. تجزیه و تحلیل بلاست نوکلئوتیدی (BLASTn) شباهت توالی نوکلئوتیدی را در حدود ۹۸,۸۵-۹۵,۱۳ بین جدایه CMV ایرانی و توالی‌های مربوطه در بانک ژن نشان داد. آنالیز فیلوژنتیک بر اساس توالی‌های نوکلئوتیدی ژن پروتئین پوششی، جدایه‌های CMV را در سه گروه اصلی گروه بندی کرد، جدایه ایرانی با جدایه‌های CMV هندی در زیرگروه IB گروه بندی شد. مطالعات بیولوژیکی با تلقیح مکانیکی یک نمونه آلوده روی توتون (*Nicotiana rustica*) انجام شد. گیاهان تلقیح شده علائم موزائیک شدیدی را در ۱۵ روز پس از تلقیح (dpi) نشان دادند و آلودگی توسط هر دو ویروس از طریق RT-PCR با استفاده از آغازگرهای اختصاصی تایید شد. این مطالعه اولین گزارش از آلودگی مخلوط گیاهان گوجه فرنگی با ToBRFV و CMV در ایران را ارائه می‌دهد.

کلمات کلیدی: آنالیز فیلوژنتیک، گوجه فرنگی، تهدید نوظهور، زیر گروه IB، آلودگی مخلوط

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Introduction

Tomato plants hold significant importance for farmers in Iran and worldwide, serving as a valuable source of nutrition and income. However, tomatoes are highly susceptible to viruses that cause substantial yield and quality losses in tomato cultivation. The rise in the number of viruses affecting tomatoes can be attributed to the globalization of agricultural trade and rapid climate change (Gautam *et al.* 2013; Menzel *et al.* 2019). Moreover, the global trade of agricultural products plays a pivotal role in facilitating the spread of plant viruses and spread of newly introduced viruses (Jones & Naidu 2019).

Among the viruses infecting tomato crops, *tomato brown rugose fruit virus* (ToBRFV) has gained significant attention due to its rapid global spread. ToBRFV belongs to the *Tobamovirus* genus within the *Virgaviridae* family. It is characterized by rod-shaped particles and a single-stranded, positive-sense RNA genome contains four open reading frames (ORFs) (Luria *et al.* 2017). In the infected plants, ToBRFV causes a range of symptoms, including mild to severe mosaic patterns on leaves, as well as the development of yellow or green spots, deformations, green grooves, and irregular brown spots on fruits that these symptoms significantly impact the marketable yield of tomatoes (Luria *et al.* 2017). Since its initial outbreak in tomato crops in Jordan (Salem *et al.* 2016), ToBRFV has rapidly spread and become a significant global threat to tomato production. Moreover, the infection of ToBRFV in pepper plants is also increasing. The swift transboundary movement of the virus across countries within just seven years of its initial report in Jordan can be attributed to the global trade of infected fruits and seeds, as well as its mechanical transmissibility. In 2021, ToBRFV was detected in greenhouse-grown tomato plants in Iran (Ghorbani *et al.* 2021; Esmaeilzadeh & Koolivand 2022), and later that year, it was also found to infect pepper plants in greenhouses (Esmaeilzadeh & Koolivand 2022).

In recent years, there have been reports of mixed infections involving ToBRFV with other viruses such as *tomato spotted wilt virus* (TSWV), *pepino mosaic virus* (PepMV), resulting in more severe symptoms and reduced yields compared to single infections (Luria *et al*. 2017; Klap *et al*. 2020). Mixed-infections with two or more plant viruses are common in nature or fields. During these mixed-infections, various types of interactions occur, which can be either synergistic or antagonistic in nature (Syller *et al*. 2012; Klap *et al*. 2020). The majority of studies have indicated that *southern tomato virus* (STV), a widely spread viral disease with high incidences in tomato plants, does not induce apparent symptoms in tomatoes when infecting them alone. However, when STV interacts with other viruses like PepMV, CMV, PhCMoV or ToMV, it may induce the severe symptoms. In the case of ToBRFV, synergistic interactions have been observed, resulting in more severe symptoms on tomato fruits (Luria *et al.* 2017). Furthermore, mixed infections provide opportunities for viral recombination between co-infecting viruses, leading to the emergence of new variants or species and new viral disease problems in the future (Lukman *et al.* 2019). Mixed infection of ToBRFV and TSWV, ToBRFV and pepper mild mottle virus (PMMoV) have previously been reported from Iran (Esmaeilzadeh & Koolivand 2023). However, specific studies on the interaction between ToBRFV and CMV in mixed infections are currently lacking in the available literature. The objective of the present study is to investigate the occurrence of mixed infections of ToBRFV with members of the *Orthotospovirus* genus, PMMoV, ToMV and CMV in tomato plants under natural greenhouse conditions. Understanding the effect of these mixed infections is crucial for comprehending the interactions between these viruses and their impact on tomato plant health. Additionally, it sheds light on the potential risks associated with the emergence of novel viral variants through recombination events.

Material and methods

Sampling

In order to monitor the outbreaks of ToBRFV in various greenhouses across Iran following its worldwide emergence and recent report in the country, surveys were conducted during the growing seasons (September 2021 to February 2022). Tomato plants exhibiting viral symptoms, including mosaic and blistering on leaves, were sampled from multiple greenhouses located in the Southwest and Northwest regions of Iran including East Azerbaijan (Julfa), West Azerbaijan (Khoy), Zanjan and Hormozgan. In addition to ToBRFV-like symptoms, the collected samples showed symptoms such as shoestring and leaf deformation which are indicative of several different tomato viruses, suggesting the possibility of mixed infections. To investigate the presence of ToBRFV and other common tomato viruses, such as members of the *Orthotospovirus* genus, PMMoV, ToMV and CMV a total of 10 tomato samples were selected for further testing.

PCR amplification and sequencing

Total RNA was extracted from 100 mg of fresh leaf tissue using the RNX Plus Kit (SinaClon, Iran). The extracted RNA was then used to synthesize first-strand cDNA using the Easy cDNA Synthesis Kit (Parstous, Iran) with random hexamer primers according to the manufacturer's instructions. To detect the presence of ToBRFV, a polymerase chain reaction (PCR) was performed with specific primers TBRFV-F-5722 (5'-CACAATCGCAACTCCATCGC-3') and TBRFV-R-6179 (5'-CAGAGGACCATTGT AAACCGG-3') (Panno *et al*. 2019) corresponding to a part of the coat protein gene. The PCR amplification was carried out with a cycling condition of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Furthermore, the possibility of mixed infection with other viruses was investigated using the universal primer gl3637-F (5'-CCTTTAACAGT(A/T/G)GAAACAT-3') and gl4435c-R (5'-CAT(A/T/G)GC(A/G) CAAG A(A/G)TG(A/G)TA(A/G)ACAGA-3') (Chu *et al*. 2001) targeting the L segment (RdRp) of orthotospoviruses with the cycling condition of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min with 72 °C for 10 min of final extension. For PMMoV, the primers used were targeting the coat protein gene, PMMoVdF258 (5'-GTAAGAGAAATGATAATAAGGGTTTG-3') and PMMoVdR (5'- CGTTCGCAAATACACGTCAC -3') (Zhou *et al*. 2021) with a cyclic condition of 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s of denaturation, 52 °C for 30 s of annealing, 72 °C for 1 min with final extension of 72 °C for 10 min. The presence of ToMV was investigated using primer pairs ToMVFor (*5′*-CTCCATCGTTCACACTCGTTACT-3′) and ToMVRev (*5′*-GATCTGTCAAAGTCTGAGAAACTT-3′) (Jacobia *et al*. 1998) with initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 30s, 62 °C for 45s, and 72 °C for 1 min, and a final elongation at 72 °C for 5 min as well as the CMV coat protein gene-specific primers CMVCPF (5'-CGGATCCATGGACAAATCTGAATCAACC-3') and CMVCPR (5'-GGCGGCCGCTCA GACTGGGAGCACCCCAG-3') under the following conditions: 94°C at 2 min; 35 cycles of 94°C at 30 s, 52°C at 30 s and 72°C at 45 s with 72 °C for 10 min of final extension were used to test the presence of PMMoV from the symptomatic tomato samples. PCR products were analyzed by agarose gel electrophoresis, visualized with SYBR Safe staining (SinaClon, Iran), and sent for Sanger sequencing (Sinuhe Biotech Company, Iran).

Phylogenetic and Recombination analysis

The obtained sequences from this study were subjected to BLASTn analysis to compare them with available sequences in GenBank. The complete CP sequences of other ToBRFV isolates and other CMV were downloaded from the National Center for Biotechnology Information (NCBI) database (Tables 1 and 2). Multiple nucleotide sequence alignments were performed using CLustalW implemented in MEGA version 11. Phylogenetic neighbor-joining (NJ) trees were constructed based on the coat protein sequences with 1000 bootstrap replicates. The best-fitting model of substitution, determined by MEGA 11 (Tamura *et al*. 2013), was JC and K2+G. Pairwise nucleotide comparisons were conducted using Sequence Demarcation Tool version 1.2 (SDT v1.2) to calculate the nucleotide sequence identity matrix. Furthermore, potential recombination signals in the selected sequences were analyzed using seven algorithms (GENECONV, Bootscan, Chimaera, MaxChi, SiScan, 3Seq, and RDP) implemented in Recombination Detection Program version 4.97 (Martin *et al*. 2015).

Mechanical transmission:

Nicotiana rustica seedlings at the 3-4 leaf stage were selected for inoculation with an infected tomato sample to investigate mixed infections. The inoculum was prepared by grinding the infected sample in 0.1 M potassium phosphate buffer (pH 7.0) and mechanically inoculated onto *N. rustica* plants that were pre-treated with carborundum. The inoculated plants were then placed in an insect-proof greenhouse with a temperature of 25°C and a photoperiod of 16:8 (light: dark) for a duration of 2 weeks. Subsequently, symptomatic leaves were collected from the inoculated plants and tested using RT-PCR with specific primers to detect the presence of the targeted viruses.

Results

Greenhouse observation, sequencing and phylogenetic relationships/analysis

Naturally infected tomato plants exhibited characteristic symptoms of leaf mosaic, leaf deformation, and shoestring (Figure 1).

Table 1. Accession numbers and characteristics of 66 ToBRFV isolates retrieved from GenBank for phylogenetic analysis.

Accession number	Host	Country	Isolate
KT383474	*Solanum lycopersicum*	Jordan	Tom1-Jo
KX619418	*Solanum lycopersicum*	Israel	TBRFV-IL
MK133095	*Solanum lycopersicum*	Germany	TBRFV-P12-3H
MK165457	*Solanum lycopersicum*	State of Palestine	Palestinian isolate
MK319944	*Solanum lycopersicum*	Mexico	TBRFV-MX
MN167466	*Solanum lycopersicum*	Italy	ToB-SIC01/19
MN549395	*Solanum lycopersicum*	Canada	Ca1B
MN549396	*Solanum lycopersicum*	Canada	Ca2
MN882011	*Solanum lycopersicum*	Netherlands	33610411
MN882013	*Solanum lycopersicum*	Netherlands	39976860
MN882016	*Solanum lycopersicum*	Netherlands	38886230_A
MN882028	*Solanum lycopersicum*	Netherlands	39070014_A
MN882030	*Solanum lycopersicum*	Egypt	39070022_A
MN882041	*Solanum lycopersicum*	Netherlands	39070153_E
MN882042	*Solanum lycopersicum*	Netherlands	39563361_A
MN882043	*Solanum lycopersicum*	Netherlands	39563361_B
MN882045	*Solanum lycopersicum*	Netherlands	39563388_B
MN882050	*Solanum lycopersicum*	Netherlands	39941596_B-2
MN882053	*Solanum lycopersicum*	Netherlands	39941641_A-1
MN882058	*Solanum lycopersicum*	Netherlands	39941668_B
MN882059	*Solanum lycopersicum*	Netherlands	39962055_A
MN882062	*Solanum lycopersicum*	Netherlands	39962442_B
MT002973	*Solanum lycopersicum*	USA	CA18-01
MT018320	*Solanum lycopersicum*	China	ToBRFV-SD
MT118666	*Capsicum annuum*	Turkey	TBRFV-Ant-Pep
MW314091	*Solanum lycopersicum*	China	6189975_2
MW314092	*Solanum lycopersicum*	Egypt	32607982
MW314094	*Solanum lycopersicum*	Egypt	33314743
MW314098	*Solanum lycopersicum*	Netherlands	33837296_2
MW314111	*Solanum lycopersicum*	Peru	36783571_2
MW314112	*Solanum lycopersicum*	Netherlands	36783668_2
MW314118	*Solanum lycopersicum*	Netherlands	38589922_1
MW314119	*Solanum lycopersicum*	Netherlands	38589922_2
MW314123	*Solanum lycopersicum*	Netherlands	39563433_3
MW314132	*Solanum lycopersicum*	Netherlands	40002350_A
MW314136	*Solanum lycopersicum*	Netherlands	40002385_A
MZ004925	*Solanum lycopersicum*	China	Y2020-3
MW314110	*Solanum lycopersicum*	Jordan	36689794_1
MZ438228	*Solanum habrochaites*	Jordan	Tom2M-Jo
MZ945420	*Solanum lycopersicum*	Belgium	GBVC_ToBRFV_02
NC_028478	*Solanum lycopersicum*	Jordan	Tom1-Jo
OK339579	*Solanum lycopersicum*	Mexico	Mex2_26r
OK624678	*Solanum lycopersicum*	Italy	Tom-BA21
OM515232	*Solanum lycopersicum*	United Kingdom	2020015323_B
OM515233	*Solanum lycopersicum*	Peru	36364500_1
OM515235	*Solanum lycopersicum*	Peru	40732089_3
OM515248	*Solanum lycopersicum*	Netherlands	38950951
OM515258	*Solanum lycopersicum*	Peru	41108421
OM515261	*Solanum lycopersicum*	Belgium	39474756
OM515264	*Solanum lycopersicum*	Netherlands	41903353
OM515269	*Solanum lycopersicum*	Netherlands	38665691
OM515270	*Solanum lycopersicum*	Belgium	33613331
OM515272	*Solanum lycopersicum*	Netherlands	41903150
OM718704	*Solanum lycopersicum*	Netherlands	41903310
OM892671	*Solanum lycopersicum*	USA	S4
OM892672	*Solanum lycopersicum*	USA	S6
OM892674	*Solanum lycopersicum*	USA	S11
OM892675	*Solanum lycopersicum*	Mexico	S15
OM892676	*Solanum lycopersicum*	Peru	S17
OM892678	*Solanum lycopersicum*	Peru	S19
OM892679	*Solanum lycopersicum*	USA	S20
OM892682	*Solanum lycopersicum*	USA	S24
OM892683	*Solanum lycopersicum*	USA	S25
OM892686	*Solanum lycopersicum*	Mexico	S28
MN882020	*Solanum lycopersicum*	Netherlands	38887559_B

Table 2. Accession numbers and characteristics of 42 CMV isolates retrieved from GenBank for phylogenetic analysis.

Accession number	Host	Country	Isolate
AY871068	Cucumber	Iran	SH17
AB042294	-	Indonesia	IA
U31219	*Musca* sp.	Hawaii	Hawaii
U20668	-	USA	Fny
AF127977	-	China	K
AB008777	-	China	SD
AM183119	*Solanum lycopersicum*	Spain	Ri-8
FJ621496	*Cucumis sativus*	Poland	Woj
D10539	-	USA	M
AJ006988	-	China	P1
M22710	-	Japan	M22710
D12499	-	Japan	Y
AB049568	-	Japan	HL
D28489	-	Japan	CS
JF327832	*Nicotiana tabacum*	China	yunyan 87
DQ002876	-	Iran	DI1
DQ002885	Cucumber	Iran	GI1
DQ002883	Squash	Iran	F13
X65017	-	China	HC210
EU414786	*Petunia hybrida*	China	ND2
AY429432	*Arachishy pogaea*	Netherlands	CA
EU414784	*Nicotiana benthamiana*	China	LW
AJ829779	*Solanum lycopersicum*	Spain	VAL90
AM183116	*Solanum lycopersicum*	Spain	Pl-1
Y10886	*Solanum lycopersicum*	Italy	Tfn
AJ829778	*Solanum lycopersicum*	Spain	BAR92
U31220	Musa	USA	Oahu
U20219	*Solanum lycopersicum*	USA	Ixora
JF279609	muskmelon	India	Bal-In
FJ168035	*Capsicum annuum*	India	Ch-Ada
X89652	*Physalis minima*	India	CP25
EF153733	*Chrysanthemum morifolium*	India	Lucknow
AF350450	*Hyoscyamus muticus*	India	L
HE583224	*Cucumis sativus*	India	Palampur
EF202597	*Solanum lycopersicum*	China	Tsh
AB368501	*Solanum lycopersicum*	Japan	PF
AF063610	-	USA	S
AF127976	-	USA	LS
EU191027	*Lonicera caprifolium*	Poland	WicDS
HM480051	*Cucurbita pepo*	Poland	C2
U10922	*Spinacia oleracea*	USA	DKRD

Figure 1. Natural and simultaneous infection of tomato plants by *tomato brown rugose fruit virus* (ToBRFV) and *cucumber mosaic virus* (CMV). Symptoms including of mosaic, shoestring, leaf deformation (Left), and blistering on leaves (Right).

A total of 10 tomato samples were selected and ToBRFV were detected in all samples. A 458 base pair fragment corresponding to the region of the coat protein gene was amplified in RT-PCR assay using ToBRFV-specific primers, (Figure 2).

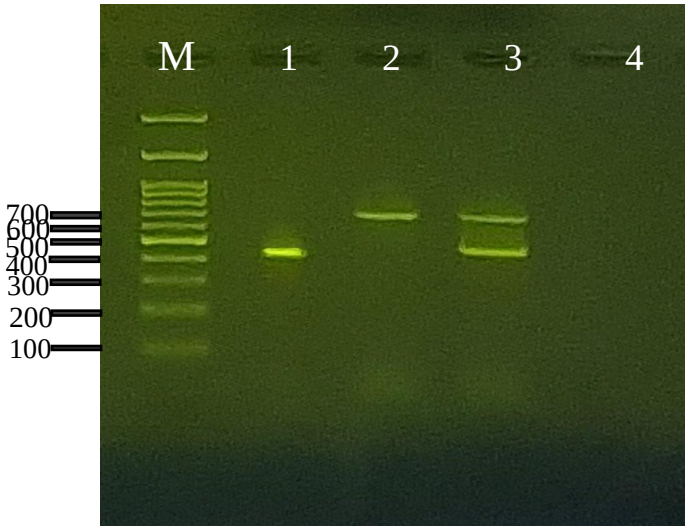

Figure 2. Electrophoresis performed to analyze the RT-PCR products amplified using *tomato brown rugose fruit virus* and *cucumber mosaic virus* specific primer pairs. Lane M: 1 kb DNA ladder (marker), Lane 1: Sample amplified with ToBRFV primers, 2: Sample amplified with CMV primers, 3: Sample amplified with simultaneously infected by ToBRFV and CMV, 4: Negative control.

One sample was sequenced and sequence BLAST analysis and multiple alignment confirmed that the Iranian isolate shared 99.76% nucleotide sequence identity with other isolates in the GenBank. The obtained sequence was deposited in GenBank under the accession number of OM807073. Phylogenetic analysis based on the coat protein nucleotide sequences revealed the clustering of ToBRFV isolates into three distinct clades, with the Iranian isolate (Jol-F-2022) grouped within clade II alongside isolates from various countries, including Mexico, Turkey, Israel, China, Peru, Canada, the USA, Jordan, the Netherlands, Italy, Egypt, the State of Palestine, Germany, and the United Kingdom (Figure 3a). While general primers for the *Orthotospovirus* genus and specific primers for PMMoV and ToMV did not yield any amplification. Using CMV specific primers, a 657 base pair fragment of the CMV complete coat protein gene was successfully amplified in five samples (Figure 2). According to this, ToBRFV isolates was detected in a mixed infection with CMV in tomato samples. Comparative analysis of CMV nucleotide sequences (OM807074) with those in the GenBank revealed sequence identities ranging from 95.13%-98.85%. Phylogenetic analysis based on nucleotide sequence of coat protein gene grouped CMV isolates into three main clades. Clades were divided into subgroups, and the Iranian isolate (Spec-F-72) was clustered in subgroup IB with Bal-In, Ch-Ada, CP25, Lucknow, and L isolates from India (Figure 3b).

Pairwise nucleotide identity matrices, generated using the Sequence Demarcation Tool (SDT v1.2), indicated that ToBRFV and CMV shared sequence similarities of 99% to 100% and 75% to 97% with other isolates, respectively. Investigation of potential recombination events using RDP4 did not detect any recombination events in the coat protein sequences.

Mechanical transmission

At 15 days post-inoculation (dpi), the inoculated plants exhibited pronounced mosaic symptoms, confirming the successful infection. Verification of the infection was achieved through RT-PCR amplification using specific primers for both viruses, as depicted in Figure 4.

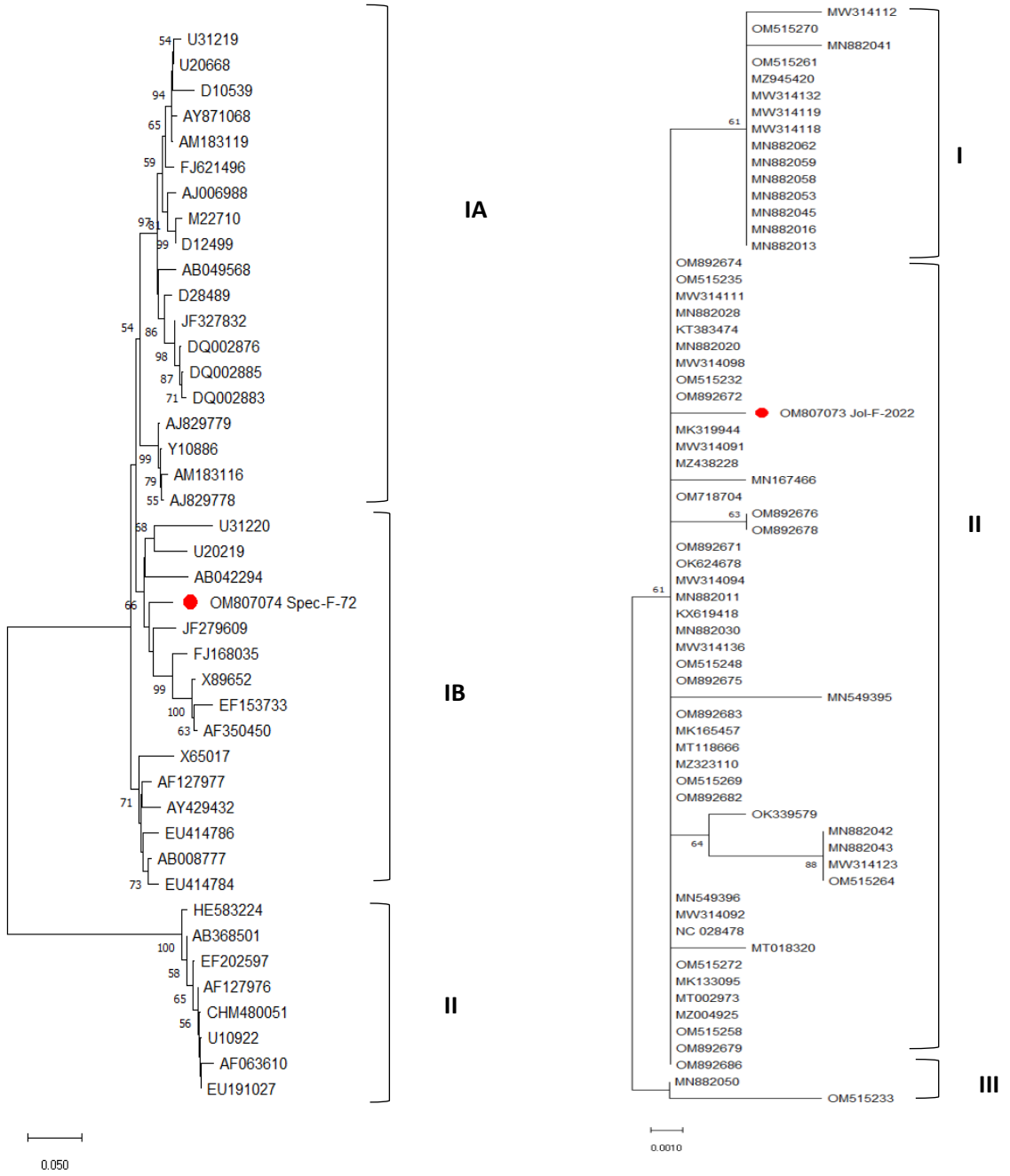

Figure 3 The neighbor joining phylogenetic trees (Distance-based) were generated to illustrate the relationship among 66 *tomato brown rugose fruit virus* (ToBRFV) isolates (Right) and 43 cucumber mosaic virus (CMV) isolates (left) based on their coat protein nucleotide sequences based on Jukes-Cantor method. Phylograms were constructed using MEGA11 with 1000 replicates. Bootstrap values (>50%) are indicated next to the branches. The Iranian ToBRFV and CMV isolates are specifically highlighted in red.

Figure 4 Severe mosaic symptoms were observed on *Nicotiana rustica* plants inoculated with ToBRFV and CMV, which appeared at 20 days post-inoculation (dpi).

Discussion

Viral diseases are major constraints on tomato production worldwide and cause significant yield and quality losses (Hansen & Lapidot 2012). Considering that many plant species can be infected by multiple viruses, finding more than one virus simultaneously in a plant is not unusual. Therefore, mixed infections can occur due to the broad host range of several viruses and the polyphagous nature of many plant virus vectors, which can transmit more than one virus to the plant. In nature, mixed infections are common and can be considered a rule rather than an exception, representing a potential source of variability due to recombination events (Scholthof *et al.* 2011; Luria *et al.* 2017; Singhal *et al.* 2021).

During a mixed infection, the involved viruses may interact with each other in a synergistic, neutral, or antagonistic manner. Precise diagnosis and comprehension of the genetic diversity and regional distribution of these viruses are vital for the effectiveness of disease management strategies. Interference with plant defense mechanisms by viral proteins is likely not the sole determinant of whether an interaction is synergistic or antagonistic: some proteins may facilitate replication, intercellular movement, and spread within the host of other viruses. For instance, in potato that can be concurrently infected by several viruses in addition to potyviruses, viral suppressor proteins of potyviruses play a role in increasing replication and enhancing plant symptoms by suppressing plant defense mechanisms against other viruses (Valli *et al.* 2018). Additional research is imperative to unravel the molecular mechanisms that underlie symptom development and interactions between these viruses in mixed infections.

In the present study, symptoms such as mosaic, leaf narrowing, and leaf distortion were observed in greenhouse tomato plants under investigation. The observed symptoms, which can be induced by various viruses including Tomato brown rugose fruit virus (ToBRFV), Tomato mosaic virus (ToMV), and Cucumber mosaic virus (CMV), were examined through molecular assays and subsequently detected viruses were sequenced. Ultimately, the simultaneous presence of ToBRFV and CMV in these plants was confirmed. The simultaneous presence of CMV and ToBRFV in tomato plants has important implications for disease management and control strategies. Out of the 10 plants examined in this study, CMV was identified in five samples, while ToBRFV was detected in all samples. The increased prevalence of ToBRFV compared to CMV in greenhouse production systems is closely associated with various modes of virus transmission, including contaminated seeds, mechanical transmission, and transmission by pollinating bees. This highlights the urgency for additional efforts in managing this virus. On the other hand, CMV by itself can induce symptoms such as leaf mosaic, leaf distortion, growth inhibition, fruit malformation, and shoestring, leading to reduced tomato plant yield. Moreover, the severity of symptoms observed in mixed infections can be more pronounced when compared to single infections with either CMV or ToBRFV alone. However, to date, no study has been conducted on the simultaneous effect of these two

viruses in experimental tomato plants. Mixed infection of ToBRFV and TSWV in pepper plants has also been reported previously (Esmaeilzadeh & Koolivand 2023). Nevertheless, their concurrent presence with ToBRFV in tomato and pepper plants and the assumption of synergistic interaction between these viruses could lead to significant losses in tomato and pepper crops yield. On the other hand, mixed infections should be considered a key factor influencing virus evolution.

Understanding the genetic diversity and evolution of CMV and ToBRFV is also crucial for developing effective control measures. Analysis of the coat protein gene of ToBRFV has revealed limited genetic diversity, which is consistent with the findings of Çelik *et al*. 2022. However, in the case of CMV, isolates exhibit high diversity and are classified into two subgroups: I and II, with subgroup I further divided into IA and IB based on the sequence homology of their genomes (Sokhandan Bashir *et al*. 2006: Sokhandan Bashir *et al*. 2008; Stanković *et al*. 2021). The coat protein gene analysis of Iranian CMV isolates indicates subgroup IB, which has been associated with East Asian origins. The regional distribution and genetic variability of CMV isolates may also impact the dynamics of mixed infections. Our findings highlight the occurrence of mixed infections in tomato plants with ToBRFV and CMV, emphasizing the importance of understanding the viral dynamics and potential challenges faced in tomato cultivation (Scholthof *et al.* 2011; Smith 2014; Singhal *et al.* 2021).

Since in a mixed infection scenario, the diagnosis and identification of viral pathogens involved and the factors controlling the spread can be complicated, it is necessary to accurately detect and understand how to improve effective strategies. Although in this study, only concurrent infection of these two viruses was examined using RT-PCR with specific primers, efforts are underway to clarify the relationships between ToBRFV and CMV, as well as the molecular mechanisms that underlie the symptom development. This research is essential for understanding virus epidemiology and laying the foundation for the development of effective management strategies.

Recently, there has been an increase in studies examining mixed viral infections. This information can serve as a rich source of valuable insights for designing control measures that go beyond merely protection and are based on the antagonistic behavior between viruses. It can involve activating plant defense mechanisms (such as RNA silencing by the first virus and then preventing or reducing damage by the second virus) to effectively manage these complex interactions.

In summary, addressing mixed infections is crucial for sustainable tomato cultivation. To gain a better understanding of the possibility and implications of mixed infections between ToBRFV and CMV, further research and investigation are necessary. These studies may involve experimental inoculations, molecular detection methods, and in-depth characterization of symptom development and viral interactions in co-infected plants.

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