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Genetic variability of two Mexican *Tomato brown rugose fruit virus* isolates and expression of symptoms in tomato and pepper

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ABSTRACT

Objective/Background. The objective was to analyze the variability of two Mexican isolates of ToBRFV after a process of inoculation and multiplication in different commercial and Mexican landraces of tomato (*Solanum lycopersicum*) (15 materials) and pepper (*Capsicum annuum*) (20 materials), and to evaluate the expression of symptoms under greenhouse conditions.

Materials and Methods. In greenhouses, the post-infection variability of two isolates was analyzed: EM-JI2021 (State of Mexico) and C-JI2021 (Colima) in 15 genotypes of tomato and 20 of pepper. Each isolate was mechanically inoculated on five plants per genotype with a total of 150 plants (56 days old) of tomato and 200 of pepper. Three plants per genotype were used as controls. Sixty-one days after inoculation, one leaf per plant was collected for RT-PCR. Incidence and symptom expression were recorded. RNA extraction was by 2% CTAB. ToBRFV-F/ToBRFV-R primers amplifying 475 bpb of the RpRd gene were used (SENASICA-CNRF). 24 RT-PCR products were sequenced, cleaned and aligned with NCBI Genbank records using MEGAv11.0.13. Based on epidemiological criteria, 34 sequences were selected from GenBank for variability analysis.

Results. Ten days after inoculation, tomato genotypes exhibited severe mosaic, mild mosaic, and reduced leaf area. In pepper, symptoms differentiated by genotype were observed, including hypersensitivity reaction, leaf deformation, stem necrosis, mosaic, yellowing, necrotic lesions, and asymptomatic condition. Between position 2,124 to 2,500 bp there was 99.74 % homology with the first

report of ToBRFV in Jordan (KT383474.1). Homology >99.74 % was found with isolates from USA (MT002973.1) and Canada (OQ674195.1). C-JI2021 exhibited no variability, while EM-JI2021 generated three haplotypes: One nucleotide change (c.2,355T>C) was detected in Mulato (pepper) and Don R (tomato), while two substitutions (c.2,278A>T; c.2,355T>C) were detected in Santawest, Altius, Sahariana and Nebula (tomato).

Conclusion. The pathogenic intensity of ToBRFV varied from asymptomatic to severe depending on the combination of host, genotype, and haplotype. In short periods of infection, three haplotypes were detected, suggesting host-dependent mutagenic capacity of the virus.

Keywords: *Solanum lycopersicum*, *Capsicum annuum*, ToBRFV, isolates.

Introduction

In Jordan, in 2015, reports were made of symptoms of brown roughness in tomato fruits (*Solanum lycopersicum*), with a 100% incidence at a greenhouse level. Etiological studies determined a new *Tobamovirus* called *Tomato brown rugose fruit virus* (ToBRFV) (Salem *et al*., 2016). It has been postulated that ToBRFV may have come from the recombination of 314 nucleotides of the region 534-848 of the replicase gene (Maayan *et al.,* 2018). The *Tomato mottle mosaic virus* (ToMMV) and the *Tobacco mosaic virus* (TMV) have been proposed as direct ancestors, with the latter being the main one (Salem *et al*., 2016). The ToBRFV genome from different countries usually display low variability, which suggests an evolutionary process from a common descendant (Salem *et al*., 2016). In addition, Maayan and collabrators (2018), through phylogenetic studies of *Tobamovirus*, concluded that ToBRFV has undergone a divergent evolutionary process with adaptation to different hosts, but with a low mutation rate over a period of 3-4 years. In general, viruses have the potential to evolve and adapt quickly under the pressure of natural selection due to high population rates resulting from the efficient intraspecies replication, the occurrence of quasispecies and the lack of genome correction mechanisms (RNA virus), which enables genetic variation and short generation times (Hanssen *et al*., 2010).

In Mexico, the ToBRFV was first reported in 2018 (Cambrón-Crisantos *et al*., 2018). This *Tobamovirus* is spread mainly seed-borne. Currently, it has been reported in 20 states of the country, including the main tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) producing entities (Camacho-Beltran *et al.,* 2019; Cambrón-Crisantos *et al*., 2018). Fruits on tomato are associated with a yellow coloration, green spot and deformation, streaking and irregular brown spots.

Mosaic, mottling and yellowing are observed on foliage. The first reports of viruses included total loss of production (Cambrón-Crisantos *et al*., 2018). Due to this, the aim of this study was to analyze the variability of two Mexican ToBRFV isolates after an inoculation and multiplication process in different commercial and native Mexican tomato (*S. lycopersicum*) (15 materials) and pepper (*C. annuum*) varieties (20 materials), and to evaluate the expression of symptoms under greenhouse conditions until the flowering stage.

Inoculation of ToBRFV. Two isolates of the virus from the State of Mexico (EM-JI2021) and Colima (C-JI2021) were used. The isolated were inoculated in a total of 15 tomato genotypes, 13 commercials and two Mexican ones, along with 20 of *C. annuum*, four varieties, 15 genotypes and one native. The isolates were performed under greenhouse conditions on 56-day-old plants. Before inoculation, Imidacloprid (1.5 mL L^{-1}) was applied as a preventive measure against vector insects. The inoculation was carried out on the second youngest leaf, by spraying carborondum followed by the phosphates buffer with the macerated tomato tissue infected with the virus (two isolates). Five plants were considered for every genotype, 150 tomato plants (for both isolations) and 200 pepper plants. The control plants (three plants per plant material) were isolated to avoid contamination. The variables evaluated were the incidence of the virus in plants and the type of symptoms in the tomato and pepper materials was recorded. Tissue was taken from young leaves 61 days after inoculation, it was photographed and preserved at -20 °C. The tissue was macerated with liquid nitrogen for molecular study.

RNA extraction and RT-PCR. The nucleic acids were extracted using the CTAB 2% method (Yu, 2012; modified by CP-LANREF, 2021). The concentrations and characteristics of the RNA were measured in the NanoDrop 2000 (Thermo Fisher Scientific 2000, USA). For the RT-PCR, primers ToBRFV-F 5-AACCAGAGTCTTCCTATACTGGGAA-3 and ToBRFV-R 5'CTCWCCATCTCTTAATAATCTCCT-3 were used, which amplify part of the small subunit of the RpRd replicase with 475 bp (SENASICA, 2018). Retrotranscription and the polymerase chain reaction (RT-PCR) were carried out in the T-100 (BioRad) thermocycler. For the retrotranscription (RT), primer R (10uM), water and 2 µL of RNA (50 ng μL^{-1}) were used to obtain a volume of 16.375 µL and it was incubated at 85 °C for 3 min. Later, to each previous reaction, the mixture of Buffer-RT (5X), dNTPs (10mM), RNAsin (40 U μL^{-1}) and M-MLV-RT (200 U μL^{-1}) was added, with a volume of 8.625 µL. The mixture was incubated at 44 °C for 60 min and 92 °C for 10 min. For the PCR, MgCl2, dNTPs, the oligos, Taq polymerase, nuclease-free water and cDNA were used in a final volume of 25 µL. The conditions were an initial denaturalization at 98 °C for 90 s,

denaturalization at 98 °C for 10 s, alignment at 55 °C for 20 s, extension at 72 °C for 40 s, final extension at 72 °C for 5 min, and finally, 72 °C (SENASICA, 2018). The PCR products were analyzed by electrophoresis in agarose gel at 1% stained with ethidium bromide and they were viewed under UV light in a photodocumenter (UVP, Biolmaging Systems, Epi Chemi II Darkroom).

Phylogenetic analysis. PCR products (24 samples) were sent to Macrogen® (Seoul, Korea) to be sequenced. The sequences (both ways) were cleaned and the ends were eliminated using the program SeqAssem (https://science.do-mix.de/software_seqassem.php). A consensus was made using the sequences to identify and compare the homology with sequences from the Genbank of the National Center for Biotechnology Information (NCBI). Sequences were chosen from complete ToBRFV genomes (three sequences) from Mexico and other countries (30 sequences) from the Genbank to perform the alignment using sequences of the fragment from the gene used in this study. The alignment was carried out using Mega 11.0.13 and Geneious 2023.0.4 (www.geneious.com) to determine the variability between sequences. The criterion for the selection of the viral sequences was according to the first reports of the virus, worldwide diversity, as well as sequences from the American continent (Table 1).

ToBRFV-related symptoms in tomato and pepper. All the commercial and native tomato material expressed symptoms 10 days after the inoculation of isolations EM-JI2021 and C-JI2021. The symptoms observed in tomato induced by ToBRFV were mainly mild to severe mosaic. A severe leaf deformation and reduction with remanence of veins was also observed (Table 2 and Figure 1). In some varieties, the clearing of veins was also observed.

Zhi-Yong *et al.* (2021) evaluated 50 tomato cultivars and no material expressed resistance to ToBRFV, showing different symptoms such as mild to severe mosaic, the formation of blisters on the leaves, necrosis in sepals and pedicels, deformation, and in fruits, yellow spots, as well as necrotic lesions with brown roughness. They also inoculated *C. annumm*, *Nicotiana benthamiana*, *N. tabacum*, *Solanum melongena* and *S. tuberosum* cv. Kexin 1, where symptoms of necrosis in inoculated leaves and stems, as well as dwarfism.

In pepper, all materials displayed a diversity of symptoms after inoculation, particularly a reaction of hypersensitivity with the falling of inoculated leaves. It was initially observed in necrotic inoculated lesions and later, the detaching of the leaf. Severe symptoms were observed in some materials such as deformation in apex leaves, stem necrosis, necrosis in veins and necrotic lesions in non-inoculated leaves (Table 2, Figure 2). These symptoms coincide with Fidan *et al.* (2021), who observed necrosis in inoculated leaves, necrotic lesions in the stem and the yellowing of leaves.

Table 1. Complete *Tomato brown rugose fruit virus* sequences obtained from the GenBank (NCBI) used for the alignment and compares with ToBRFV sequences from the study.

No. of accession	Base pairs	Host	Plant tissue	Location
MZ945420.1	6379	*Solanum lycopersicum*		Belgium
OQ674194.1	6374	S. *lycopersicum*		Canada
OQ674195.1	6242	*S. lycopersicum* cultivar Yari		Canada
OM515230.1	6375	*S. lycopersicum*		Netherlands
OM515231.1	6373	*S. lycopersicum*		United Kingdom
OM515232.1	6373	*S. lycopersicum*		United Kingdom
MZ323110.1	6394	*S. lycopersicum*		Jordan
MK648157.1	6388	*Capsicum annuum*		Jordan
KT383474.1	6393	*S. lycopersicum*		Jordan
MN882030.1	6379	*S. lycopersicum*		Egypt
MN882031.1	6379	*S. lycopersicum*		Egypt
MW349655.1	6379	*C. annuum* cultivar Tampiqueno		Mexico
OM515233.1	6369	*S. lycopersicum*	Seeds	Peru
OM515258.1	6376	*S. lycopersicum*	Seeds	Peru
OM515256.1	6361	*S. lycopersicum*	Seeds	China
MT018320.1	6392	*S. lycopersicum*		China
OM515237.1	6377	*S. lycopersicum*	Seeds	Israel
OM515234.1	6371	*C. annuum*	Seeds	Israel
OM515257.1	6367	*S. lycopersicum*	Seeds	Israel
OM515266.1	6364	*S. lycopersicum*	Seeds	Israel
OM515250.1	6371	*S. lycopersicum*	Seeds	Israel
OM515261.1	6376	*S. lycopersicum*	Fruit	Belgium
OM515265.1	6375	*S. lycopersicum*	Fruit	Belgium
MN815773.1	6354	*S. lycopersicum*		Greece
OM305070.1	6386	*S. lycopersicum*		Switzerland
MT107885.1	6386	*S. lycopersicum*		Turkey
OM515234.1	6371	*C. annuum*	Seeds	Israel
OM782671.1	6356	*S. lycopersicum*		Mexico
OM892675.1	6384	*S. lycopersicum*	Imported fruit	Mexico
OM892676.1	6381	*S. lycopersicum*	Seeds	Peru
OM892677.1	6392	*S. lycopersicum*	Store fruit	USA
OM892678.1	6393	*S. lycopersicum*	Imported fruit	Peru
OM892679.1	6375	*S. lycopersicum*	Store fruit	USA
OM892681.1	6357	*S. lycopersicum*	Leaf	USA

Variability in ToBRFV isolates. Out of the total of tomato and pepper genotypes inoculated, five pepper samples were sent to inoculate, one with C-JI2021 and four with EM-JI2021, along with 19 tomato samples, six with EM-JI2021 and 13 with C-JI2021. The sequences in the study were aligned with 80.2% of the total of the fragment of the partial RpRd gene (475 pb). The sequences displayed an identity with accessions of the GenBank with a homology that fluctuates between 99.74 and 100%, including the sequence of the virus originally described by Salem *et*

Table 2. Symptoms pf *Tomato brown rugose fruit virus* in commercial and native tomato and pepper materials expressed under greenhouse conditions.

Name	Crop	Symptoms	Name	Crop	Symptoms
Santawest	Tomato[y]	MM	Conga	Pepper[z]	A
Citali	Tomato	MM, LF, LR	Fascinato	Pepper	A
IR143466	Tomato	SM, LF, LR	Felicitas	Pepper	NLNI
Sicilia	Tomato	SM, LF, LR	Botaron	Pepper	A, SN
Sahariana	Tomato	SM, LF, LR	Godzilla	Pepper	NLNI
Altius	Tomato	SM, LF, LR, GI SM, LF, LR, CV	Kathia	Pepper	A
Don R	Tomato	MS, LF, LD	Almuden	Pepper	LD, NLNI, DAA, SN, NLN
Nebula	Tomato	MM, LF	Bachia	Pepper	LNI
Volcano	Tomato	MM, LF, CV	Cannon	Pepper	A
Ametrino	Tomato	MM, LF, CV	Gina	Pepper	NLNI, SN
Angelle	Tomato	SM, LF, LD, CV	Confidaro	Pepper	A
Olmeca	Tomato	SM, LF, LR			
UAM-X	Tomato	SM, LF, LR, CV	Cayenne	Pepper	DAA, NL
Rio Grande	Tomato	SM, LF, CV			
Totolapan, Mor.	Tomato	MM	mulato	Pepper	A
Cavanna	Pepper	A	Serrano	Pepper	SM, GI
Shir	Pepper	A	Zongolica	Pepper	MM, CR
Orangela	Pepper	A	Chile pasilla	Pepper	A
			Manzano-Ver	Pepper	A

[y]Tomato. MM: mild mosaic; SM: Severe mosaic; LF: Leaf deformation; LD: Leaf deformation; LR: Leaf reduction; CV: Clearing of veins; GI: Green islands.

[z]Pepper: A: Asymptomatic; MM: mild mosaic; SM|: Severe mosaic; LD: Leaf deformation; NL: necrotic lesions on leaves; NLNI: necrotic lesions on non-inoculated leaves; SN: Stem necrosis; DAA: Deformation of apical area; NLN: necrosis in leaf nervations; GI: Green islands; CR: Concentric rings.

al. (2016) (Table 3, Figure 3). The sequences that displayed an identity of 99.74 and 99.75% were samples inoculated with EM-JI2021 (Table 3). All the sequences were aligned with accessions from the USA and Canada (Table 3). Five sequences with an identity of less than 100% were aligned with OQ6741195.1 from Canada and one sequence from the USA (MT002973.1).

According to the analysis of the alignment based on the complete genome with 34 ToBRFV sequences and the partial genome of the replicase of the 24 sequences of this investigation, variability was observed at the nucleotide level in six sequences obtained from plants inoculated with EM-JI2021. Two changes were observed: in nucleotides c.2,278A>T and in c.2355T>C (Figure 3). The sequences under study were aligned between the positions 2,124 and 2,500 corresponding to the gene of the replicase. The nucleotide substitutions were also observed in accessions MW349655.1 (pepper tissue), OQ674195.1 (tomato leaves), and

Figure 1. Symptoms in commercial and native tomato material inoculated with *Tomato brown rugose fruit virus*. Symptoms of mild mosaic (IR143466, Nebula, Ametrino, Rio Grande, Citlali, Altius); severe mosaic, deformation and clearing of main leaf nervations (Volcano, Criollo-X and Angelle); healthy plant (Angelle control).

OM515234.1 (pepper seeds), which correspond to Mexico, Canada and Israel. However, in another investigation, no mutations of ToBRFV were found after an inoculation process in tomato and pepper (Eichmeier *et al.*, 2023). These results coincide with a study that reports between 2 and 39 nucleotide substitutions at the level of the complete genome (Abrahamian *et al*., 2022). Although the nucleotide changes are low, these changes could eventually lead to variability in the pathogenic and/or epidemiological behavior of the virus. This is supported with observations on tomato plants inoculated with the isolation from the State of Mexico (EM-JI2021), which displayed a greater recorded height and stem diameter than the plants inoculated with the isolation from Colima (C-JI2021) (Data not shown). Symptoms were also observed in tomato plants inoculated with isolation C-JI2021, which were associated to European symptoms and more severe than those caused by the isolate from the State of Mexico.

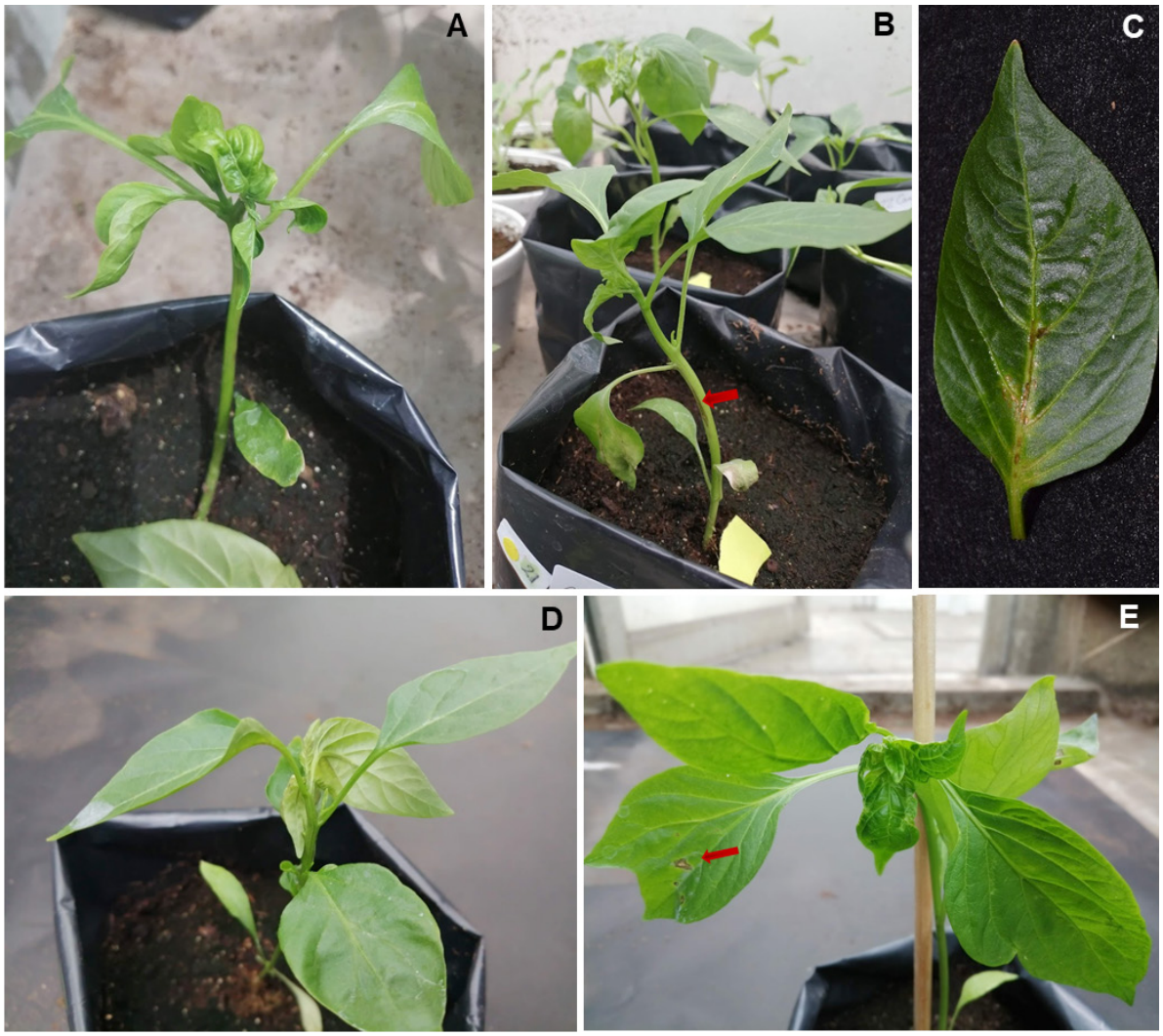

Figure 2. Symptoms in chili (*Capsicum annumm*) inoculated with *Tomato brown rugose fruit virus*. A-C) Symptoms of apical deformation, necrosis in stem and nervations of Almuden leaves; D) Symptoms of apical deformation, slight necrosis in Cayenne nervations; E) Symptoms of apical deformation, mosaic, necrotic lesions in non-inoculated Felicitas leaves.

The importance of ToBRFV, from an early diagnosis of the virus to the impact on the production of tomato and pepper, is relevant. Studies have been performed on RNA extraction methods for an early and accurate diagnose in the molecular analysis (Zamora-Macorra *et al*., 2023), up to alternatives for the management of ToBRFV with a low environmental impact, such as the use of *Beauveria peruviencis*, *Trichoderma longibrachiatum* and *Pseudomonas* sp. (Ramos-Villanueva *et al*., 2023). Nevertheless, studies on the expression of symptoms in

Table 3. Percentage of coverage and identity of two ToBRFV isolations inoculated in a total of 35 tomato and pepper genotypes under greenhouse conditions.

Isolate	Variety / creole[z]	Crop	Isolate origin	length pb	Coverage	Identity	Homolaged isolate	
							Accession	Country
EM-JI2021	Almuden	Pepper	State of Mexico	398	100	100	OQ674195.1	Canada
	Mulato	**Pepper**	**State of Mexico**	**390**	**100**	**99.74**	**OQ674195.1**	**Canada**
	Congo	**Pepper**		**391**	**100**	**99.74**	**MT002973.1**	**USA**
	Gina	Pepper	State of Mexico	381	98	100	MT002973.1	USA
	Santawest	**Tomato**	**State of Mexico**	**402**	**100**	**99.75**	**OQ674195.1**	**Canada**
	Gotzilla	Tomato	State of Mexico	383	100	100	MT002973.1	USA
	Altius	**Tomato**	**State of Mexico**	**385**	**100**	**99.74**	**OQ674195.1**	**Canada**
	Don R	**Tomato**	**State of Mexico**	**407**	**100**	**100**	**OQ674195.1**	**Canada**
	Sahariana	**Tomato**	**State of Mexico**	**392**	**100**	**99.74**	**OQ674195.1**	**Canada**
	Nebula	**Tomato**	**State of Mexico**	**408**	**99**	**99.75**	**OQ674195.1**	**Canada**
C-JI2021	Manzano	Pepper	Colima	402	100	100	MT002973.1	USA
	Santawest	Tomato	Colima	410	100	100	MT002973.1	USA
	Altius	Tomato	Colima	407	100	100	MT002973.1	USA
	Sicilia	Tomato	Colima	411	100	100	MT002973.1	USA
	IR143466	Tomato	Colima	418	100	100	MT002973.1	USA
	Don R	Tomato	Colima	408	100	100	MT002973.1	USA
	Ametrino	Tomato	Colima	384	100	100	MT002973.1	USA
	Olmeca	Tomato	Colima	399	100	100	MT002973.1	USA
	Nebula	Tomato	Colima	404	100	100	MT002973.1	USA
	Xochimilco	Tomato	Colima	384	100	100	MT002973.1	USA
	Angelle	Tomato	Colima	399	100	100	MT002973.1	USA
	Río Grande	Tomato	Colima	408	100	100	MT002973.1	USA
	Totolapan	Tomato	Colima	388	100	100	MT002973.1	USA
	Citlali	Tomato	Colima	385	100	100	MT002973.1	USA

[z] Names in italics are native materials. In bold, sequences with a homology of <100%.

the different materials available in the market, as well as knowing the variability of ToBRFV that exists on the field, provides a guideline to better understand the virus epidemiology and its behavior according to the crop and the established weather conditions. It is therefore important to study the virus diversity in both the field and greenhouse over time and to observe the association between the diversity of expression of symptoms in tomato and pepper.

	Partial sequence/bp	2,120	2138						2,130	2,278			2,290				2329					2,355				2362			2422				2447			2464		2486				2500						
		T	A	G	C	G	G	A		A	G	C	A	T	C	T	C	**G**	A	C	C	T	A	G	C	G	A	A	G	T	A	T	T	G	C	G	G	T	A	T	G	G	T	C	T	T	G	A	T
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	Altius_C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Don R_C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Sahariana_C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Figure 3. Alignment of the partial sequence of the tomato brown rugose fruit virus replicase gene from 24 sequences of isolates obtained from 35 genotypes inoculates with isolates EM-JI2021 and C-JI2021, and from 34 sequences from different tomato and chili-producing countries. The alignment was performed using Geneious.

Conclusions

All tomato materials expressed symptoms of mosaics, but without showing symptoms as severe as those reported for the first time, except in tomato plants inoculated with isolation C-JI2021. In pepper, lesions were recorded on inoculated leaves (hypersensitivity reaction), as well as necrotic lesions on the stem, apical deformation, necrosis in leaf nervations and asymptomatic.

According to the alignment analysis of two ToBRFV isolates (State of Mexico and Colima), in comparison with 34 isolates available in the NCBI Genbank, in five sequences of isolates from the State of Mexico (EM-JI2021) the substitution of two nucleotides were observed, nucleotides c.2,278A>T and c.2355T>C. Accessions MW349655.1 (Mexico), OQ674195.1 (Canada) and OM515234.1 (Israel) coincided with isolate EM-JI2021 (inoculated with the variety Don R) in one nucleotide change c.2355T>C. In this study, a total of three haplotypes were found. This is the first study that reports variability of ToBRFV in a short period under controlled conditions. This information may be relevant for cross-protection studies.

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