THE TOMATO BROWN RUGOSE FRUIT VIRUS IS RESTRICTED TO SPECIFIC AREAS IN SONORA, MEXICO - A STUDY OF 2021-2022 SEASON †

[EL VIRUS CAFÉ RUGOSO DEL TOMATE ESTA RESTRINGIDO A ÁREAS ESPECÍFICAS EN SONORA, MÉXICO - UN ESTUDIO DE LA TEMPORADA 2021-2022]

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SUMMARY

Background: Tomato brown rugose fruit virus (ToBRFV) has spread worldwide affecting tomato crop production in several countries. Although there are commercial ToBRFV-resistant tomato varieties; still, careful monitoring of its prevalence is necessary for establishing feasible epidemiological control programs. Since Sonora has a strategic geographic location for tomato cultivation and export, studies on the detection and prevention of ToBRFV outbreaks are pertinent as part of the actions to achieve the eradication of this virus. **Objective:** To determine the recent prevalence of ToBRFV in tomato-producing areas of the state of Sonora, Mexico in the period 2021-2022, using a combination of molecular and serological tools. **Methodology:** Samples of tomato leaves and fruits were collected from commercial greenhouses in different regions of Sonora from September 2021 to June 2022. Forty-four tomato leaves and 42 seed samples were analyzed for ToBRFV using reverse transcriptase-polymerase chain reaction and by direct detection of viral epitopes by immunochromatography. **Results:** 60% and 48% of the samples tested positive for ToBRFV by immunochromatography and RT-qPCR, respectively, showing an exact agreement regarding the samples' origin. **Implications:** Future research may focus on genomic analysis of viral isolates that would provide clues of ToBRFV propagation speed and emergence of particular viral strains typical of the place of origin. **Conclusion:** The presence of ToBRFV in the state of Sonora, Mexico is limited to specific geographic sites, which suggests that local regulations and phytosanitary actions have worked to control the spread of the virus.
Key words: ToBRFV; Tobamoviruses; Sonora; Tomato.

RESUMEN

Antecedentes: El virus café rugoso de tomate (ToBRFV) se ha propagado por todo el mundo afectando la producción de cultivos de tomate en varios países. Aunque, ya existen variedades comerciales de tomate resistentes a ToBRFV; todavía, es necesario el seguimiento cuidadoso de su prevalencia para establecer programas de control epidemiológico viables. Dado que Sonora tiene una ubicación geográfica estratégica para el cultivo y exportación de tomate, los estudios sobre la detección y prevención de brotes de ToBRFV son pertinentes como parte de las acciones para lograr la erradicación del virus. **Objetivo:** Determinar la prevalencia reciente de ToBRFV en zonas productoras de tomate del estado de Sonora, México en el periodo 2021-2022, utilizando una combinación de herramientas moleculares y serológicas. **Metodología:** Se recolectaron muestras de hojas y frutos de tomate de invernaderos comerciales en diferentes regiones de Sonora desde septiembre de 2021 hasta junio de 2022. Se analizaron cuarenta y cuatro muestras de hojas de tomate y 42 de semillas para detectar ToBRFV mediante la RT-PCR y por detección directa de epítopos

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virales por inmunocromatografía. **Resultados:** El 60 % y el 48 % de las muestras dieron positivo para ToBRFV por inmunocromatografía y RT-qPCR, respectivamente, mostrando una concordancia amplia con respecto al origen de las muestras. **Implicaciones:** La investigación a futuro puede centrarse en el análisis genómico de aislados virales que proporcionen pistas sobre la velocidad de propagación de ToBRFV y la aparición de cepas virales particulares típicas del lugar de origen. **Conclusión:** La presencia de ToBRFV en el estado de Sonora, México, se limita a sitios geográficos específicos, lo que sugiere que las regulaciones locales y las acciones fitosanitarias han funcionado hasta cierto punto para controlar la propagación del virus.
Palabras clave: ToBRFV; Tobamovirus; Sonora; Tomate.

INTRODUCTION

Among the members of the *Tobamovirus* genus, tomato brown rugose fruit virus (ToBRFV) stands out for attacking plants of agroeconomic interest such as tomatoes and peppers (García-Estrada *et al*., 2022). Since its appearance and first reports in Jordan (Salem *et al*., 2015), ToBRFV has been detected in new geographical areas such as Spain (Alfaro-Fernandez *et al*., 2021), Syria (Hasan *et al*., 2022), Norway (Hamborg and Blystad 2022), France (Skelton *et al*., 2022), China (Yan *et al*., 2019), and Saudi Arabia (Sabra *et al*., 2022). This rapid and progressive spread has positioned ToBRFV as a gradual threat to tomato cultivation worldwide (Oladokun *et al*., 2019). In Mexico, the presence of ToBRFV has been reported in the states of Michoacán (Cambrón-Crisantos *et al*., 2019), Baja California Sur (Camacho-Beltrán *et al*., 2019), and Yucatán (Magaña-Álvarez *et al*., 2021). However, the Mexican phytosanitary regulation agency (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria; SENASICA) reported ToBRFV-positive cases in at least 15 different states (SENASICA, 2019).

The state of Sonora has a strategic position in tomato production for two main reasons; the first one is its geographical proximity to the USA, which absorbs more than 90% of Mexican tomato exports, and the second is related to geo-climatic and territorial situations shared with the neighboring states Sinaloa and Baja California, which are both among the largest producers of vegetables in Mexico (Zamora, 2016; SAGARHPA 2018). Thus, Sonora is positioned as one of the 10 states with the highest tomato production at the national level and according to the Servicio de Información Agroalimentaria y Pesquera (SIAP), four Sonoran regions (Valle de Yaqui, Obregón, costa de Hermosillo, and Magdalena de Kino) are cataloged as historical in the cultivation and export of tomato (SIAP 2020).

Although it was thought that the presence of *Tm*-1, *Tm*-2, and *Tm*-2^2 genes in *S. lycopersicum* commercial varieties constituted an effective defense system against all tobamoviruses that infect tomato (Chanda *et al*., 2020), the appearance of ToBRFV, that cannot be contained by the expression of *Tm*-2^2, changed the current world panorama for this crop. Although ToBRFV-resistant tomato varieties already exist (Fernandez, 2022; Rijk Zwaan Group, 2022); they are just beginning to enter the commercial market and become known among producers. Timely detection of ToBRFV outbreaks is conducted by inspection of leaves and peduncles of suspicious plants with apparent infection and is confirmed by immunochromatography or molecular analysis such as RT-PCR, which allow producers to estimate a relative degree of damage and risk.

According to the most recent data from the Food and Agriculture Organization of the United Nations (FAO 2020), tomato production exceeds 180 million tons worldwide, while economic losses in important crops caused by viruses surpass 30 billion dollars per year (Yang *et al*., 2021). However, there are no reliable reports about losses caused year after year by ToBRFV infections. A feasible phytosanitary program aimed to contain the spread of pathogens should comprehend timely epidemiological surveillance studies that allow the analysis of the current situation and facilitate its control and resolution. Therefore, the objective of this work was to determine the recent prevalence of ToBRFV in tomato-producing areas of the state of Sonora, Mexico in the period 2021-2022, using a combination of molecular and serological tools.

MATERIAL AND METHODS

Plant material

Solanum lycopersicum L. sampling was conducted *ad hoc*, looking for characteristic symptoms of tobamoviruses infection such as yellow spots and/or rough skin appearance on the fruit, or mosaic patterns and wilting of leaves. All samples were obtained from commercial tomato greenhouses (the names of the participating companies are confidential and will remain anonymous) with cultivable area dimensions between 10,000 to 15,000 m^2, from different municipalities in the state of Sonora, Mexico (Figure 1). In the period between September 2021 to June 2022, leaf and mature fruit samples of Saladette tomato crops were collected in sterile bags, labeled, transported to the laboratory, and stored at -80 °C until analyzed.

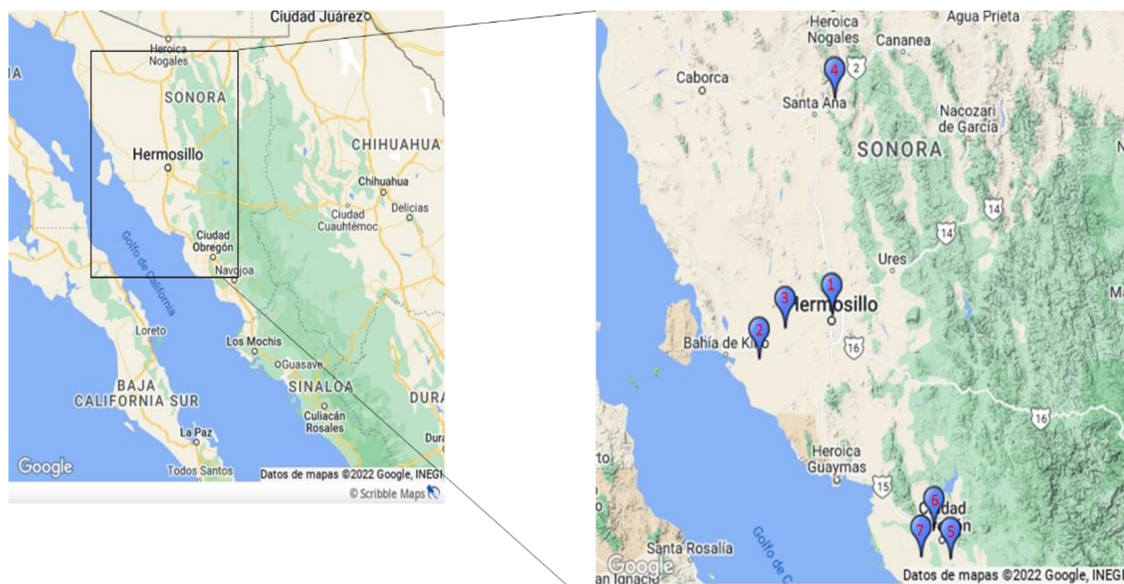

Figure 1. Geographic representation of sampling sites for ToBRFV screening in the state of Sonora, Mexico. 1) Hermosillo, 2) Bahía de Kino, 3) Miguel Alemán, 4) Magdalena de Kino, 5) South Obregón City, 6) North Obregón City, and 7) Bácum.

Extraction of viral RNA

Total RNA extraction was performed by grinding samples in liquid nitrogen and subsequent use of Invitrogen's Concert™ Plant RNA Reagent following the manufacturer's instructions. The integrity of isolated RNA was verified in 1% agarose gel electrophoresis, and quantification was performed in a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific).

Screening of the *Tobamovirus* genus by end-point PCR and constitutive gene control

For the initial detection of tobamoviruses in tomato samples collected in the state of Sonora, viral RNA was retrotrascribed using SuperScript™ II Reverse Transcriptase kit with the reverse primer R-4718 (5'-CAATCCTTGATGTGTTTAGCAC-3') previously reported by Luria *et al*. (2017). Briefly, end-point PCR was performed in 25 µL (final volume) reactions containing 0.5 µL of correspondent cDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 25 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 mM of both forward (F-3666: 5'-ATGGTACGAACGGCGGCAG-3') and reverse (R-4718) primers, and 2U of *Taq* DNA polymerase (GoTaq Flexi DNA polymerase, Promega, USA). PCR was carried out in a MJ Research PTC-200 thermal cycler according to the following cycling conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1.5 min and finally one cycle of 72 °C for 10 min. The amplification of a 1,052 bp product was expected. Amplicons were resolved on 1% agarose gels and visualized in a UV light transilluminator (ImageQuant LAS 4000, GE Health care Bio-Sciences AB).

For the amplification of the constitutive gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), cDNA synthesized with an oligo (dT) primer and the SuperScript™ II Reverse Transcriptase kit from 1000 ng total RNA was used as template in an end-point PCR assay. Primers for *GAPDH* amplification (LeGAPDHF1 5'-GCCAAGAAGGTTGTGATCTC-3' and LeGAPDHR1 5'-TCTTCCACCTCTCCAGTCC-3') were designed with the Primer3 software using specific *S. lycopersicum* sequences. End-point PCR was performed in 25 µL (final volume) reactions containing 0.5 µL of correspondent cDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 25 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 mM of both forward and reverse primers, and 2U of *Taq* DNA polymerase (GoTaq Flexi DNA polymerase, Promega, USA). PCR was carried out in a MJ Research PTC-200 thermal cycler according to the following cycling conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min and finally one cycle of 72 °C for 10 min.

ToBRFV detection by real-time PCR

For real-time PCR amplification, 10 ng of cDNA (total RNA equivalents) previously synthetized with the reverse primer ToBRFV-R-6179 (5'-CAGAGGACCATTGTAAACCGG-3'; Panno *et al*., 2019), were used as template in 21 µL final volume reactions of iTaq Universal SYBR Green Supermix kit (BIORAD), with 0.5 µM of forward primer ToB5520F

5’-GTAAGGCTTGCAAAATTCGTTCG-‘3 (genomic position: 5496-5520) and 0.5 µM of reverse primer ToB5598R 5’-CTTTGGTTTTGTCTGGTTTCGG-3’ (genomic position: 5598-5575), (both primers do not present specific hybridization with other organisms, and were described by Panno *et al*., 2019). Amplification conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The ToBRFV positive control consisted of viral cDNA isolated from ToBRFV-infected leaves kindly donated by Laboratorio de Ciencia y Tecnología de Productos Agrícolas para Zonas Tropicales y Subtropicales from CIAD-Culiacán. PCR grade water was used for non-template reactions instead of cDNA and all reactions were run in triplicates in a 48-well StepOne Real-Time PCR equipment (Applied Biosystems) using ROX dye (50 µM) as internal fluorescence reference (Thermo Scientific). Dissociation melting curves were performed at the end of each qPCR assay to confirm the presence of a single amplification product.

ToBRFV detection by immunochromatography

For the detection of ToBRFV by immunochromatography, ImmunoStrip® reactive immunostrips, Agdia (ISK 66800/0025) were used following the manufacturer’s procedure. For this, all the samples for each sampling site were pooled and analyzed. Briefly, all test strips have two reaction lines, the top one appears as long as the reaction is valid. The bottom line appears only when the sample is positive for the presence of ToBRFV, if the sample is negative this reaction line does not appear.

RESULTS

A total of 86 samples from 44 tomato plants (a leaf sample and a seed sample from fruit were obtained from 42 plants, while two leaf samples were obtained from two additional plants that did not have fruits), were obtained from 7 different greenhouses dedicated to tomato cultivation in the state of Sonora: Hermosillo (15 samples), Bahía de Kino (3), Miguel Alemán (12), Magdalena de Kino (4), South Obregón (24), North Obregón (3), and Bácum (25). Four samples out of the 42 tomato fruit samples collected had pericarps with evident characteristic traits of tobamovirus infection. However, these fruits did not present necrotic lesions or internal alterations in color and smell.

The amount of RNA obtained from leaves and seeds presented differences in quantity and quality. Due to the nature of seeds, it was more difficult to obtain abundant yields of RNA from this source. However, *GAPDH* amplification in all samples indicated that total RNA was of suitable quality for retrotranscription and for the detection of tobamovirus sequences by PCR (Figure 2).

Subsequently, the detection of viral sequences by end-point RT-PCR in tomato leaves and seeds showed that only 5 leaves of 25 samples from Bácum and 2 leaves of 4 samples from Magdalena de Kino presented the 1,052 bp amplicon corresponding to viruses belonging to the *Tobamovirus* genus. The rest of the samples (*n* = 57, both leaves and seeds) did not present the corresponding amplicon and were considered negative.

Real-time PCR detected ToBRFV sequences in 48% (*n* = 41/86) of tested samples. Table 1 describes the disposition of the ToBRFV-positive samples by location. The positive control showed a Ct value of 19±5, whereas Ct values for ToBRFV-positive samples ranged from 14-22. Also, two samples corresponding to Bahía de Kino presented high Ct values between 28 and 29 units, suggesting a low viral transcript abundance. Finally, no evident differences were found in the Ct values of infected samples because of the type of sample, either leaves or seeds, that showed mean Ct values of 21±9 and 19±3, respectively.

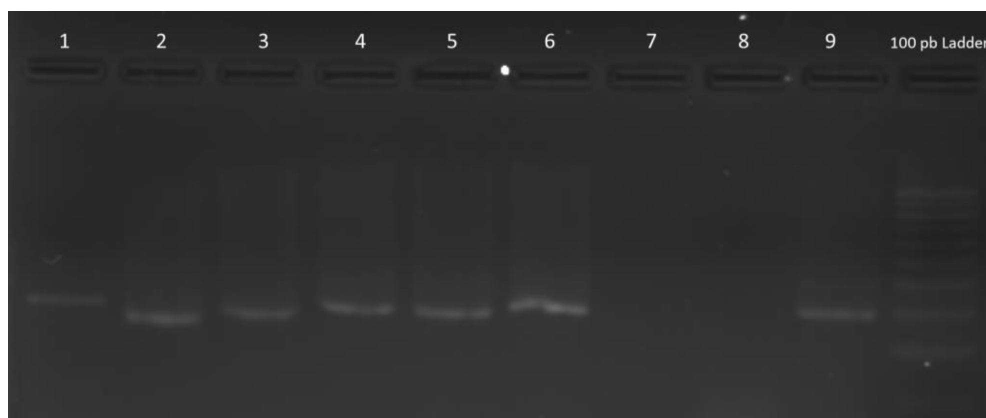

Figure 2. End-point PCR for *S. lycopersicum GAPDH* detection. Samples 1 to 6, positive for 265 bp amplification of *S. lycopersicum GAPDH*. Samples 7-8, negative controls. Sample 9, positive control, and finally 100 bp ladder.

Table 1. Comparison between ToBRFV-positive samples detected by molecular and biochemical tools.

Localities	Total number of samples	End-point PCR	Immunochromatography	qPCR (mean Ct value)
Miguel Alemán	12	0	Negative	0 (Undetermined)
Bahía de Kino	3	0	Negative	3* (21± 7)
South Obregón	24	0	Positive	18 (19±3)
North Obregón	3	0	Negative	0 (Undetermined)
Bácum	25	5	Positive	17 (20±5)
Magdalena de Kino	4	2	Positive	3 (14± 3)
Hermosillo	15	0	Negative	0 (Undetermined)
Total	86	7	52	41

*Bahía de Kino showed two positive samples with high Ct values, indicative of low viral loads. Immunochromatography tests were performed on pooled samples.

Through immunochromatography, it was detected that composite samples from three localities were positive for ToBRFV. These results suggest that 60% of the samples (n = 52/86) were ToBRFV-positive, originating from sampling sites in Magdalena de Kino, South Obregón, and Bácum.

DISCUSSION

The presence of ToBRFV in tomato crops threatens the production and economy of the region. Due to high mechanical transmission of tobamoviruses and considering previous reports from government surveillance agencies on the presence of ToBRFV in the state of Sonora, our study expected to find a high percentage of ToBRFV-infected samples. However, our findings suggest that ToBRFV-eradication brigades conducted jointly by the federal government and local producers have worked to contain the spread of the virus. Sanitary records of the presence of ToBRFV in Sonora date from the 2018-2020 period, where export tomato samples from Sonora and Sinaloa were found ToBRFV-positive (SINALAB, 2020). Although, in the present study the presence of ToBRFV in the state of Sonora was found circumscribed to specific geographic regions, the viral prevalence represents a latent risk due to its ability to spread. Hence, our findings can help raise awareness and establish control measures for the management of solanaceous crops in these regions. In this sense, the affected greenhouses were notified confidentially, and specialized advice was offered for the integrated management of phytopathogens when required.

As one of the strategies followed in the present study, the RT reaction was primed with a specific reverse primer for identifying the tobamovirus genus. Under this methodology, we were only able to identify 7 (8%) positive samples out of 86 evaluated, which may suggest a lower sensitivity of this approach. In an attempt to describe our finding, we expected high Ct values for these 7 samples (e. g., greater than 25). However, by means of real-time PCR they presented Ct like the other positive samples.

Analyzing the three approaches used, the immunochromatography technique showed comparable results with those obtained by real-time PCR. However, the end-point PCR approach presented poor performance, probably due to the fact that in conventional PCR detection amplified products have to be abundant enough to be detected through agarose gel electrophoresis and densitometric means. On the other hand, three ToBRFV-positive samples collected from Bahía de Kino, which were not detected by the immunochromatography method, displayed high Ct values (>28) by qPCR, suggesting they had low viral loads and therefore were unable to show a strong agglutination reaction to be detected by the immunostrip system. The use of TaqMan probes is recommended to improve sensitivity and specificity in ToBRFV detection, however a cost-benefit analysis should be taken into consideration.

In this work, almost half of the samples evaluated were positive for the presence of ToBRFV. A recent study near our area reported a transmission rate of 9% for ToBRFV through various greenhouse substrates (Vargas-Mejía *et al*., 2023) and in another study also conducted in Mexico, it was shown that ToBRFV may be present in all tomato-producing areas in the country (García-Estrada *et al*., 2022; Gonzalez-Concha *et al*., 2021). Although there is no real estimate on how this viral disease impacts the economy, it is established as a widely prevalent disease, with a complex approach that negatively impacts the agroeconomic yield of the tomato production. Only in USA through a survey of growers reported economic losses of approximately 17 million dollars annually attributed solely to ToBRFV infections (IUNU team members, 2022).

From the classical and most accepted point of view, mechanical transmission by seeds and contaminated work material are the main routes of spread of viruses (Davino *et al*., 2020; Salem *et al*., 2022 b). However,

recent studies have found new reservoirs and transmission possibilities (Ilyas *et al*., 2022). For instance, Salem *et al*., (2022 a) identified the presence of ToBRFV in up to eight different plant families, suggesting that crop rotation might not be enough to rule out the risk of future infections. Additionally, new niches and hosts may play a predominant role in the transmission-infection of ToBRFV in *S. lycopersicum*. There is an open possibility of ToBRFV transmission by pollinating insects, as reported by Levitzky *et al*., (2019) for *Bombus terrestris*. On the other hand, samples obtained in a brief time after plantation could suggest a prominence of seed transmission, whereas an incidence higher later in the season could suggest a natural dispersion. Under this assumption, the results in this work were obtained from mature tomato plants that almost finished their production cycle, supporting the hypothesis of a natural dispersion of viral particles.

At least two different approaches are required for a reliable detection of viral phytopathogens. The strength of this study lies in the robustness of biochemical and molecular methodologies that allowed the identification, at the transcript and protein levels, of ToBRFV in *S. lycopersicum* grown in the state of Sonora, Mexico, which advances the knowledge on the current panorama of the risk of spreading ToBRFV. Future research may focus on genomic analysis of viral isolates that would provide clues of ToBRFV propagation speed and emergence of particular viral strains typical of the place of origin.

CONCLUSION

The ToBRFV was detected in leaves and fruit samples from tomato greenhouses in different locations in Sonora, Mexico, with nearly half of the samples analyzed in this study showing a positive result. The cases of ToBRFV were restricted to specific places, which shows that government and producer actions to control the virus do have an effect.

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Conflict of interests. The authors declare there are no competing interests.

Compliance with ethical standards. This work not involved human subjects. Therefore, does not require approval by an ethical committee

Data availability. Data are available writing to Dr. Miguel Ángel Martínez Tellez upon reasonable to norawa@ciad.mx

Author contribution statement (CrediT). M. Sánchez-Sánchez- methodology, writing - original draft**; E. Aispuro-Hernandez**- formal analysis, investigation**; E.A. Quintana-Obregón**- writing - review and editing, visualization**; M.A. Martínez-Tellez**– conceptualization, funding acquisition, supervision**.** All authors have read and agreed to the published version of the manuscript

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