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A ToBRFV similar to a European isolate in a Mexican greenhouse, its presence in seeds and its inactivation for virus integrated management.

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Abstract

Tomato brown rugose fruit virus (ToBRFV) has acquired a general interest after being reported in at least twenty-three countries, causing concerns in the tomato global agro-industry. Therefore, different aspects of integrated management (disease identification, sources of infection, and sanitation procedures) are of paramount importance. Here we report the identification and molecular characterization of a ToBRFV isolate, its genome sequence, and the second one, isolated in Mexico, similar to isolates from the Netherlands and the Middle East. After infection of plants with this viral isolate, we detected its presence in seed coats and derived seedlings at a very low frequency. Furthermore, we could isolate virus-like particles (VLPs) from greenhouse water obtained from wells albeit these particles were not so infective as a crude extract from an infected plant. We also tested the chemical and thermal inactivation of ToBRFV as monitored on *Nicotiana rustica* detached leaf bioassays.

Introduction

Tomato brown rugose fruit virus or ToBRFV belonging to the *Martellivirales* order, *Virgaviridae* family, and *Tobamovirus* genus, was first detected in Israel and Jordan five and six years ago[1, 2]. In the Americas, the first occurrence was reported in 2019 in the United States and Mexico in 2019 [3–5]. Its widespread occurrence is due, among other things, to its efficient mechanical transmission and seed-borne condition. No full demonstration of seed transmission through the embryo exists to this date. However, suggestions are that the virus on the outer seed coat can infect the seedling. A report about the absence of the virus inside the plant embryo suggests its presence in the seed endosperm [6]. ToBRFV can be easily transmitted vertically and mechanically to other plants. Therefore, implementing seed and greenhouse sanitation measurements is a common practice [7]. Due to the stability of the virus on the seed coat and contaminated surfaces and tools, disinfectants and phytosanitary measures are recommended. The question arises as to which chemical or physical treatment is the more feasible to use per region, beneficial, and easy to implement to treat greenhouse surfaces [6, 7]. If they are to be used for effective virus biological inactivation, a quick method to evaluate the chemical or physical treatments is highly desirable.

ToBRFV has spread globally since 2016, with at least twenty three countries reporting the disease in different journals: eleven countries in Europe (Germany, Greece, Italy, Netherlands, Turkey, United Kingdom, Spain, France, Albania, Switzerland and Norway); three in North America (Canada, United States, and Mexico); six in Asia (Israel, Jordan, Palestine, Syria, Saudi Arabia and China); one in South America (Peru), and one in Africa (Egypt) [8–15]. In addition to tomatoes, the virus infects Capsicum annuum [4, 15, 16]. Under laboratory conditions, species such as Petunia spp., Solanum spp., and Nicotiana spp. are susceptible to ToBRFV [1]. As with other new diseases, plant health bulletins and local forums are standard practices to exchange experiences on preventive measures to mitigate the spread of the virus, which can quickly happen through tools, substrates, and other materials [17]. The pollinating bumblebees used inside the greenhouses can also be vector-like agents [18].

In Mexico, tomato is produced mainly under greenhouse conditions, and to avoid a large outbreak of ToBRFV best management practices are recommended for greenhouse production. In general, these practices include a survey of the source of seed used and sanitation, targeting the reduction of transmission and disease spread while still being safe for plants and workers [3, 19]. To investigate some sources of contamination and critical practices for sanitation and management of ToBRFV under greenhouse conditions, in this paper we performed a stepwise approach. First, we isolated ToBRFV from an infected plant and characterized its genomic sequence. We then identified its phylogenetic relationship with other ToBRFV sequences available on NCBI. We then implemented diagnostic assays, such as RT-PCR from isolated virus-like particles (VLPs) or total RNA, to detect virus contaminating tomato seed coats, their derived epicotyl seedlings, water from greenhouse wells, and new or recycled substrate slabs. Finally, we evaluated the inactivation of ToBRFV, for greenhouse sanitation purposes, after chemical and thermal treatments using quick bioassays on detached leaves of *N. rustica* (Fig. 1). We obtained evidence of seed-borne transmission after surveying the presence of ToBRFV in seeds from infected plants. We compared the efficacy of chemical and heat treatments for sanitization using detached leaf bioassays.

Materials And Methods

Samples

Ten tomato plants showing leaf mosaics, wrinkling, and deformations from a commercial greenhouse with an incidence of 10% infection with ToBRFV, were collected in one growing season in 2018 and kept under freezing conditions until we used leaves for virus diagnosis. Also, we sampled ten substrate slabs having a composition of 50% coco peat + 50% husk chips, with original 15–18% humidity. For seeds, we used commercial (labeled as resistant to ToMV, with Tm-2(2) gene), and seeds derived from five ToBRFV infected plants, to obtain seed-washings or epicotyls for VLPs extractions. We used seeds from commercial anonymous sources and fruits collected from ToBRFV-infected plants. For water samples we collected either one liter of 100 mL from six water sources from greenhouses with covered-medium technology, drip irrigation system, no recycling of residual water, and conventional management practices. We used sterile double distilled water as a negative control.

Seed washing, seed germination and seedling sampling

We separated lots of batches of about 900 (ToMV resistant, with Tm-2(2) gene) commercial seeds into 300 seeds per assay for external washing and for germination. We placed the seeds for external washing in an Eppendorf tube, with the addition of 1200 µL of PBS buffer at pH 7.4, followed by a 5–20 min vigorously agitation. The washing solution was transferred to a new tube and centrifuged for 10 min at 5,000 rpm for VLPs extraction.

Seeds for germination were washed with 15% sodium hypochlorite and germinated in batches of 50 on small glass flasks on sterile filter paper at 28 °C with a 12h light-dark photoperiod. After 18 days of germination, epicotyls were separated from the seed coat at the middle portion of the germinated seedling stem and used for VLPs extraction.

We inoculated ToMV-susceptible tomato plants (carrying the Tm-2(2) gene) for seed testing from infected plants with ToBRFV inoculum obtained from a control plant (NS0 plant). We confirmed infection by RT-PCR and collected seeds for seed washings and used lots of 60 seeds. After washing, the seeds were disinfected and surveyed for remanent virus using RT-PCR.

Extraction of VLPs from leaf, seedlings, seed washings and water source samples

We ground five grams of leaves from symptomatic plant samples or seedlings in liquid nitrogen and placed them in a 50 ml Falcon tube, adding 40 ml of 1x phosphate buffer (PBS) pH 7.4 and shaking thoroughly. Subsequently, 125 µl of Triton X-100 and 70 µl of 0.25 M iodoacetamide were added and mixed to homogenize. We then centrifuged the mixture at 5,000 rpm for 60 min at 4°C, and recovered the supernatant centrifuged at 13,000 rpm for 20 min at 4 ° C. We recovered the supernatant and filtered it sequentially in a 0.45 µm and in a 0.22 µm pore sized filters. We then added 10% (v/w) of polyethylene glycol (PEG 8,000) to the double-filtered solution, mixed thoroughly, and incubated at 4°C overnight, followed by centrifugation at 13,000 rpm for 60 min at 4°C. We used the pellet for resuspension with 2 ml of 1x PBS buffer (pH 7.4) and transferred it to an Eppendorf tube with one volume of chloroform. The mixture was mixed thoroughly and incubated at room temperature for 15 min, followed by centrifugation at 13,000 rpm for 10 min at 4°C. We washed again with chloroform recovering the aqueous phase and storing it at -20°C until use. For water extraction of VLPs, we filtered the samples directly into the 0.45 µm and 0.22 µm pore-sized filters, and then PEG precipitated as described before. We quantified the concentration of VLPs using a 1:10 dilution in PBS, of which 0.25 µl was placed in a copper grid coated with formvar and dried at room temperature, and added 7 µl of 2.5% uranyl acetate to the grid and incubated for 5 min. We removed the excess uranyl acetate with a filter paper and observed the samples in transmission electron microscopy (TEM).

RNA extraction from VLPs

In an Eppendorf tube with 200 µl of a solution containing VLPs, we added 1 µl of RNAse A, 1 µl of DNAse, and 2 µl of 10x PCR Buffer with 25 mM MgCl2 (1:1). We incubated the reaction for 2 hours at 37°C and then added 4 µl of Proteinase K, 16 µl of 10% SDS, 2 µl of 1M Tris-HCl pH 8.0, 4 µl of 0.5M CaCl2, and incubated for 20 min at 50°C. We then added 200 µl of phenol and 200 µl of chloroform and centrifuged at 13,000 rpm for 10 min at 4°C. We transferred the aqueous phase to a new Eppendorf tube; then, we added one volume of phenol and centrifugated again. We transferred the upper aqueous phase to a new tube and precipitated the RNA with 32 µl of ammonium acetate at 5M and 400 µl of absolute ethanol for 2 hours at -20°C. Subsequently, we centrifuged the tubes at 13,000 rpm for 5 min at 4°C; discarded the supernatant and washed the pellet with 400 µl of 70% ethanol. Finally, the tube with the washed precipitate was centrifuged at 12,000 rpm for 5 min at 4°C and then dried to resuspend in 15 µl of dd water.

RNA extraction from plants and plant substrate

We followed two protocols for RNA extraction from plants, substrate slabs, and seeds. The first is based on the TRIzol ™ method, used for cDNA synthesis and viral genome sequencing. The second followed the protocol of the PureLink RNA Mini Kit ™ kit. Here, the protocol was modified: the lysis and homogenization steps were done with 1 ml TRIzol and 200 µl chloroform per 100 mg of the substrate.

RT-PCR for cDNA synthesis

We used the reverse transcriptase enzyme ReverseAid H minus RT (Thermo Scientific), with the extracted RNA as a template for cDNA synthesis in a tube with 10 µL of water and 2 µL of RNA, adding 1 µL of the different reverse oligonucleotides to ToBRFV per reaction (Table SI1). The tube was incubated at 65 ° C for 5 min and placed on ice for one minute, then 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTPs each, and 0.2 µl of RevertAid H Minus Reverse Transcriptase were added. Finally, we incubated the reactions at 42°C for 1 hr and 10 min at 72°C. We took one µl of cDNA to amplify the viral fragments, with Taq polymerase (Thermo Scientific, Waltham, MA, USA) at 5 U/µl, 10X Taq Buffer, 25 mM MgCl2, 2 mM of each dNTPs. PCR was set up with different steps, according to the set of primers used, on an Arktik, MiniAmp Plus thermocycler from Thermo Scientific (Waltham, Massachusetts, USA).

Chemical and thermal treatments

We used eight chemical treatments to inactivate ToBRFV: phosphoric acid (61%), lime (calcium hydroxide 75%) and glutaraldehyde plus ammonium quaternary salts (0.3%) (G + S), NaOH at 0.8 and 1%, glutaraldehyde at 1%, pentapotassium salts at 1% and benzoic acid at 4%. We prepared all the solutions with sterile distilled water. In all cases, we mixed 500 µl of ToBRFV inoculum (described above for plant S0) and 500 µl of the chemical treatment solution in Eppendorf tubes and homogenized by inversion with a further incubation at room temperature for 10 minutes. We mixed the positive control virus inoculum with 1x PBS and used only PBS solution as a negative control. We inoculated the control and treated ToBRFV in detached *N. rustica* leaves bioassays and inspected them daily after 5 to14 days.

We also tested three temperatures (thermal treatments) for virus inactivation: 87, 92, or 97°C for 15 minutes of incubation at 300 rpm in an Eppendorf tube Thermo Mixer of crude extracts derived from 100 mg of S0 plant tissue after resuspension in 500 µl of inoculation buffer after centrifugation at 3000 rpm for 15 minutes. We used 6 detached leaves per inoculum heat treatment in two different bioassays using detached *N. rustica* leaves. For the positive inoculum control, we used 1 gram tissue of plant NS0 and resuspended it in 500 µL of 0.5 M phosphate buffer.

Leaf bioassays using *N. rustica* detached leaves

We weighed 1 gram of tissue from plant NS0, resuspended it in 500 µL of 0.5 M phosphate buffer and used it for all chemical and thermal treatments, followed by leaf bioassays. After testing *N. benthamiana* and *N. tobacco*, we decided to use *N. rustica* plants as a source for inoculation on leaf bioassays. In this species, ToBRFV produced prominent symptomatic leaf spots, and leaves were turgent through the observation period in the growing chamber compared to detached leaves from *N. benthamiana*. Followed

by inoculation into detached *N. rustica* leaves, in the left upper corner, we placed them in a tight-closed plastic box as a moisture chamber and daily registered for the appearance of viral yellow spots indicative of a virus infection.

Sanger sequencing and phylogenetic analysis

We used amplicons for Sanger sequencing derived from total RNA (for partial sequences) or VLPs from plant NS0 (genome sequence) with the oligos listed in Table S1. We aligned the nucleotide reads to the ToBRFV reference genome KT383474 and annotated the ORFs with the Geneious prime software.

We retrieved available near-complete genome sequences of ToBRFV from NCBI for phylogenetic analysis and aligned them with Clustal Omega under default parameters. Model selection for phylogeny reconstruction was performed with SMS [20, 21], showing that the GTR + I model was the most suitable for the maximum likelihood phylogeny reconstruction, performed with PhyML 3.0 with a bootstrap of 500 [20, 21].

Results

Plant for the source of inoculum and positive control

After spotting tomato plants with mosaics symptoms in commercial net greenhouses in the West of Jalisco, Mexico, we screened symptomatic plants using RT-PCR with specific primers directed towards a segment of the replicase protein (RdRp) or the vicinity of the coat protein and the 3'UTR (CP-3'UTR) (Fig. 2a). Plants and seed samples, water wells, and substrate slabs from the greenhouses were analyzed using extracted virus-like particles (VLPs) and observed under the transmission electron microscope (TEM, Fig. 2b). RT-PCR from VLPs or total RNA led us to confirm the identity of the putative infecting virus. One symptomatic tomato plant (S0) tested positive for ToBRFV (plant S0), used for genome sequencing, was the positive control for all tests in this work. We used a crude extract from this infected plant as a viral inoculum for infection and chemical or thermal viral inactivation tests. We estimated about 360 million particles per gram of tissue of this infected plant.

ToBRFV-Mx2 genome has a higher similarity to isolates from Netherlands

We amplified a near full-length genome of 6265 nt, from VLPS isolated from plant S0, comprising the four reported OFRs [2], and recovered both 5' and 3' end of the virus (Figure SI 1, SI 2 and GenBank: OK339579) partially. This new Mexican isolate (ToBFRV-Mex2) shows more similarity with isolates from the Netherlands (MW314133, MW314134, MW314135) [22] than with the first Mexican ToBRFV (MK319944) published and the other North American genomes (Fig. 3).

ToBRFV in seed coat and seedlings

As an insight into possible contamination of seeds (seed-borne virus), we extracted VLPs from seed washes and epicotyls derived from washed, disinfected, and germinated seeds. We used seeds from ten anonymous commercial brands (S1 to S10) and five ToBRFV-infected tomato plants (S12, S13, S14, S16, and S18). We extracted RNA from VLPs to confirm the presence of ToBFRV in seedcoats and epicotyls (Table 1) and found it in the seed coat of three commercial samples (S1, S7, and S8), suggesting contamination in the outer part of the seed. S1 and S7, two of those samples, also had ToBRFV in the epicotyl, while S8 tested positive only on the seed coat. Five tested positives for only the epicotyl (S3, S5, S6, S10, and S1). For seeds derived from five ToBRFV-infected tomato plants (S12, S13, S14, S16, and S18), we detected the virus in only the washing of one batch (S14). Washed seeds from this batch were germinated or further disinfected and analyzed for virus presence after seed grinding. We found ToBRFV presence in seed washing, ground seeds, and epicotyls, which suggests virus presence not only seed-borne but also its transmission from infected plants.

Table 1
ToBRFV detection from VLPs extracted from commercial and infected plants after seed coat-washings and epicotyls.

Batch	Seedcoat	Epicotyl	Seed origin
S1	+ [a]	+ [a]	Seeds from commercial sources
S2	-	-	
S3	-	+ [a]	
S5	-	+	
S6	-	+	
S7	+ [a]	+ [a]	
S8	+ [a]	-	
S9	-	-	
S10	-	+	
S12	-	-	Seeds from infected plants
S13	-	-	
S14	+	+	
S16	-	-	
S18	-	-	

[a] Partial Sanger sequencing

ToBRFV amplicons and VLPs of water samples and substrate slabs

Understanding possible sources of virus contamination help implement sanitation measures inside a greenhouse. For this purpose, we tested samples from water sources (wells) and substrate slabs (which contain coconut fiber) for the presence of ToBRFV, where plants with viral symptoms were growing. We extracted VLPs from twenty water sources (wells) and tested them for ToBRFV by RT-PCR. We found four water sources (W1, W2, W4, and W5) positive for ToBRFV (Fig. 4), suggesting those wells represent a source of viral contamination. However, VLPs from these sources were not as infectious in detached leaf bioassays or inoculated in plants compared to the S0 inoculum. We only observed one infected leaf out of 6 inoculated in two leaf-detached bioassays.

To evaluate the capacity of three disinfection treatments, we carried out bioassays using detached leaves of *N. rustica* inoculated with ToBRFV isolated from plant S0. This bioassay allowed us to use multiple leaves from one Nicotiana plant to visualize viral symptoms at 5–7 dpi. We tested the efficacy of eight disinfectants solutions inactivating the virus inoculum: phosphoric acid (61%), lime (calcium hydroxide 75%), glutaraldehyde plus ammonium quaternary salts (0.3%) (G + S), NaOH at 0.8 and 1%, glutaraldehyde at 1%, pentapotassium salts at 1% and benzoic acid at 4%. Out of the eight chemicals tested, G + S and pentapotassium salts (PS) were the most effective, with no observed viral symptoms at the inoculation site but chemical damage at 14 dpi. Lime treatment was the most inefficient disinfectant as 8 of 12 leaves presented infection at 14 dpi. After phosphoric acid treatment, 5 out of 12 leaves showed symptoms (Fig. 5, Table 2).

Table 2
Presence of ToBRFV symptoms in control and disinfectant treated leaves bioassay at 14 dpi.

Treatment	Inoculated leaves	Symptomatic leaves	%
- Control	12	0	(0%)
+ Control	12	12	(100%)
G + S	12	0	(0%)
Lime	12	8	(66%)
Phosphoric acid	12	5	(41%)

We also used thermal treatments to assess the biological inactivation of ToBRFV inoculum and in substrate slabs. We treated the ToBRFV inoculum at 87, 92, and 97 ℃ for 15 and or 30 minutes and infected detached leaves of *N. rustica*. Treatments at 92 and 97 ℃ for 30 min effectively inactivate ToBRFV (Table 3). Inactivation at 87 ℃ was the most inefficient as the virus could infect all the leaf inoculated areas in two detached leaf bioassays. For virus detection in slabs, we initially mixed substrate from slabs with 10% tomato leaves infected with ToBRFV to make sure we could detect the virus in these samples. These inoculated slabs resulted negative for the virus, suggesting a possible interference of coco peat in the slabs with RNA extraction, we therefore, used a kit (see Materials and methods) for RNA

isolation that resulted in virus detection Using the inoculated ToBRFV slabs as positive controls, we found no amplified virus amplicons on duplicates of six thermally treated substrate slabs at 95 ℃, suggesting these were not sources of virus contamination for the plants inside the greenhouses.

Table 3
Biological inactivation of ToBRFV as estimated by lesions in leaf bioassays after virus inoculum exposure at different temperatures

Viral inoculum treatment	Exp 1	Exp 2
15 min at 87 ℃		
	5/5	5/5
15 min at 92 ℃	3/5	2/5
30 min at 92 ℃	0/10	0/10
15 min at 97 ℃	1/5	0/20
30 min at 97 ℃	0/10	0/10
No temperature exposure	5/5	10/10
No virus	0/13	0/3

Discussion

Understanding and controlling possible sources of virus contamination depends on different factors, for instance, identifying the virus infecting the plants, the source of seeds used and, to a certain extent, an efficient method to evaluate virus inactivation methods after sanitation. We identified ToBRFV in greenhouses form Jalisco state and sequence the genome from one sample. The ToBRFV-Mex2 genome sequence generated grouped with isolates from the Netherlands, and this group was within a more extensive clade conformed with isolates from the Middle East, Peru, and Egypt. ToBRFV-Mex2 isolate reported here did not group with the isolate from Mexico (MK 19944). Despite certified seeds, the phylogenetic analysis suggests multiple introductions of ToBRFV into the country. It is worth mentioning that the Netherlands is a large distributor of seeds in Mexico, and ToBRFV-Mex2 groups with isolates MW314133, MW314134, MW314135. In 2019, an outbreak of ToBFRV sustained the possible assumption of the Netherlands being the possible source of origin for this isolate. Those newly reported sequences from Europe form part of the present repertoire of ToBRFV genomes in the Nextstrain platform [11, 22].

Even though no clear-cut evidence has been given as to how seed contamination occurs, the differential presence of VLPs in seed coat and not in epicotyls and vice versa supports the possible presence of the virus in the inner part of the seed. The presence of ToBRFV as a seed-borne virus derived from infected plants also supports the idea of its seed transmissibility at a low frequency. However, studies using in situ hybridization would give more solid support to the mechanisms of seed transmission of ToBRFV

Bioassays using plants to discern between the effects of chemicals and or temperature for virus inactivation can be time-consuming [7, 23, 24]. In this study, we selected detached leaves of *N. rustica* over other Nicotiana species (results not shown) for bioassays estimating ToBRFV infectivity. This type of bioassay allowed us to evaluate the biological inactivation of the virus as early as five days post-inoculation by identifying local chlorotic spots produced in the vicinity of the inoculated leaves. We compared this system with the inoculation of leaves in whole plants of *N. bethamiana, N. tabacum*, and *N. rustica* in the greenhouse and detached leaves in growing chambers. The latter allowed us to detect viral symptoms in fewer days, with apparent symptoms testing more conditions in a smaller space. We standardized our method by using the same amount of tissue from an infected plant (sample S0) with an equivalent number of virions for each bioassay.

The chemical treatments that inactivate the virus are glutaraldehyde, quaternary ammonium salts (G + S), and pentapotassium salts. These chemicals are considered practices of integrated strategies for virus mitigation [25, 26]. Our results indicated that treating plastic ware, substrates, and other greenhouse utensils and tools for 30 minutes at 92 ℃ inactivates the virus (ToBRFV). Heat treatment could be implemented effortlessly as a routinary phytosanitary measurement.

We could not detect ToBRFV amplicons from recycled heat-treated substrates slabs where tomatoes are grown inside the greenhouse. Substrate from slabs seems to interfere with detecting the virus by RT-PCR using conventional RNA extraction based on Trizol. Water contamination and high mechanical transmissibility of the virus might be essential factors contributing to the spread and need consideration in phytosanitary programs. However, isolated VLPs from water were not as biologically active as the crude extract from infected plants. They caused less or no symptoms in the detached leaf bioassays. One possible explanation is a possible inactivation during the extraction protocol of VLPs.

It is of paramount importance to consider the possible sources of infection during the exponential phase of infection, where an increase in the disease-transmission rate takes place [27, 28].

A precise scenario of the movement of genomic sequences in seeds or other means will become more evident as more genomic sequences become available on platforms like Nexstrain [22] which will occur in this considered rapidly spreading plant virus on tomato plants [29]. On the meantime, more testing of commercial sources of seeds, along with more genome sequencing, in random assays should be routinely considered as most of the tomato seeds used inside commercial greenhouse come from only few countries.

Declarations

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Author Contributions: Conceptualization of the work was done by Laura Silva-Rosales and Emmanuel Ávila de Dios. The experimental work was done by Irving J. García-López, Daniel A. Salas-Aranda, Ruth S. Pérez-Alfaro and J. Natzul. Santoyo-Villa. Software and data analysis was made by Pablo. Vargas-Mejía

and Ricardo I. Alcalá-Briseño. The first draft of the manuscript was written by Pablo Vargas-Mejía and Ricardo I. Alcalá-Briseño. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

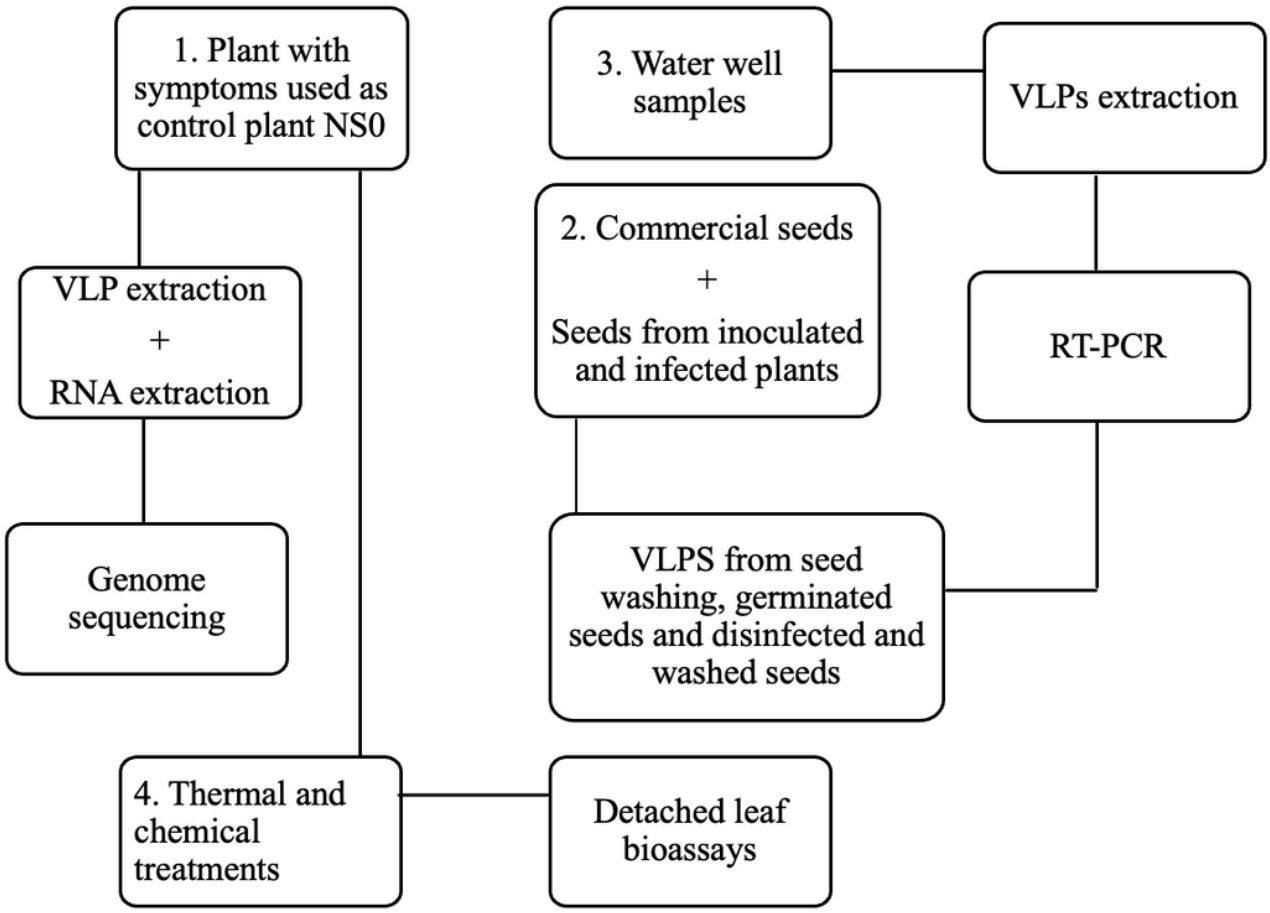


Figure 1

Flow chart of used strategy to detect, sequence, and test for seed borne ToBRFV

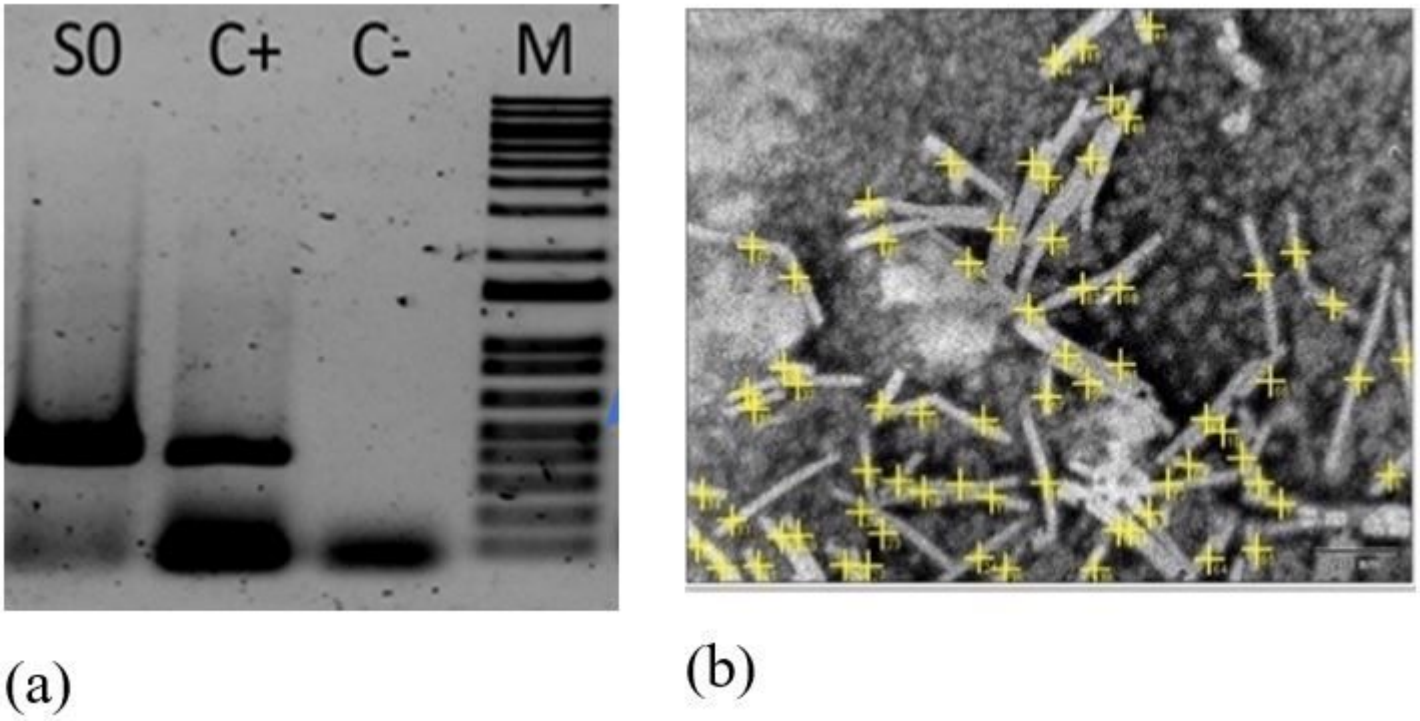


Figure 2

ToBRFV amplicons and VLPs from one plant (S0) (a) Amplicons from the ToBRFV replicase of 476 bp obtained from VLPs of the source plant S0 and from a previously tested positive sample (C+); (b) VLPs (69 virions marked with yellow +) in a section of a TEM grid to estimate viral load per gram of tissue obtained from the plant S0

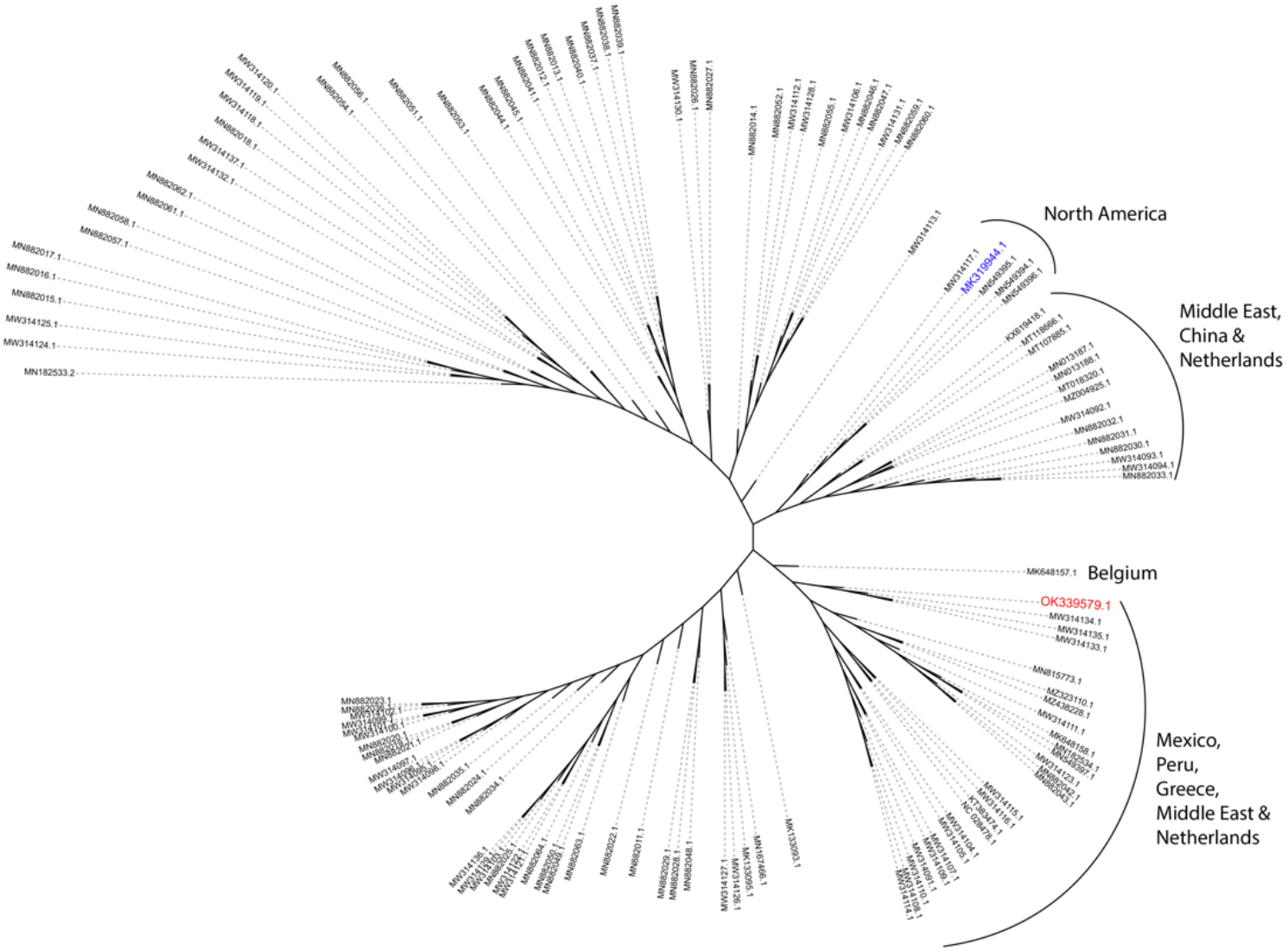


Figure 3

Unrooted phylogenetic tree of genomic sequences of ToBFRV, showing the new Mexican isolate ToBFRV-Mex2 (in red), reported in this study. Blue writing shows the Mexican isolate ToBFRV-Mex1. One hundred twenty-six near full-length ToBRFV genomic sequences were aligned and used to construct a maximum likelihood tree with the GTR+I model

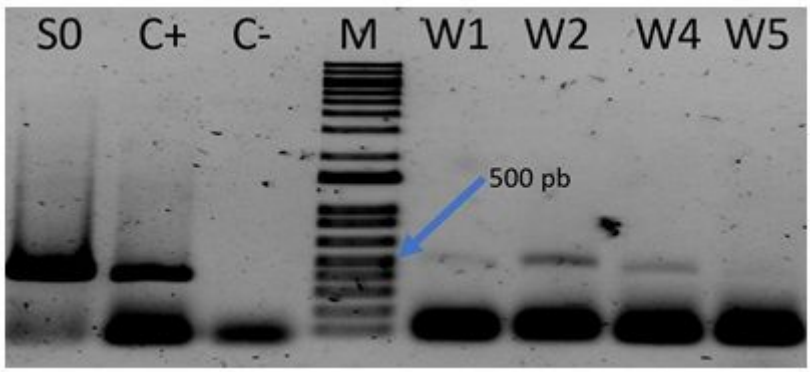


Figure 4

ToBRFV amplicons derived from VLPs extracted from water sources (a) Amplicons from the ToBRFV from VLPs of three water sources (W1, W2, W4, and W5)

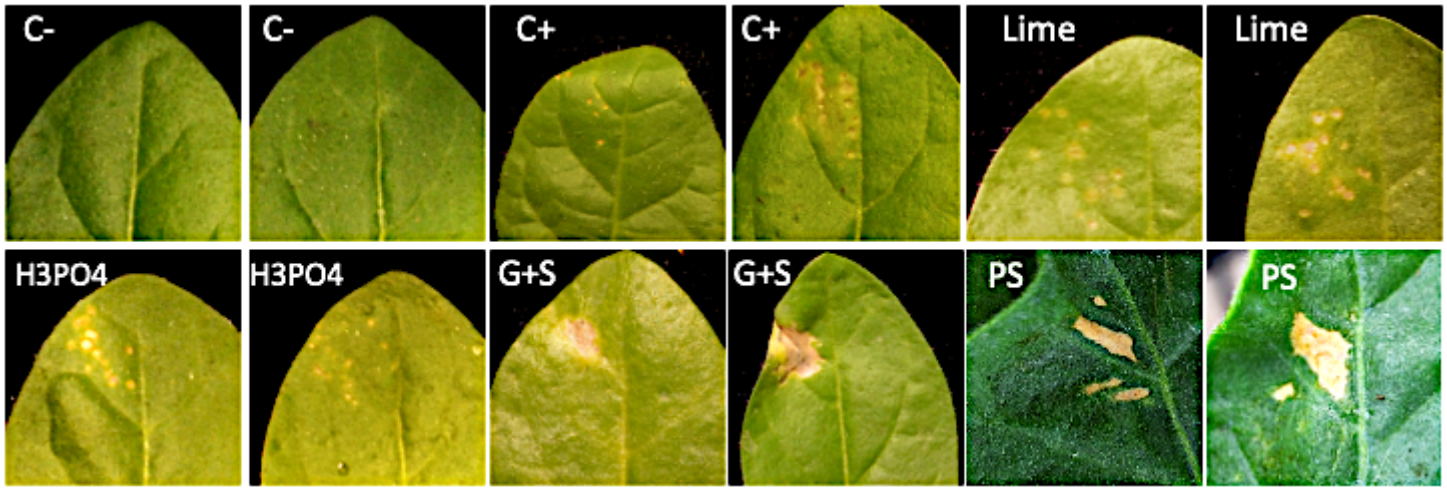


Figure 5

ToBRFV necrotic circular spots produced on N. rustica detached leaves on a moist chamber after 7 days post-inoculation with viral inoculum from plant S0 and treated with lime (upper right panel); phosphoric acid (lower central panel) and G+S (lower central panel). The chemical produces necrosis in G+S treatment and PS as no RT-PCR amplicons were obtained in leaves from this treatment

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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