



Studying tomato brown rugose fruit virus longevity in soil and virion susceptibility to pH treatments helped improve virus control by soil disinfection

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Abstract

Background and aims Tobamoviruses are highly stable soil-borne pathogens posing a challenge to a monoculture practice. Biochemical and physical properties of tobamovirus virions were studied by analyses of tobacco mosaic virus (TMV). Little is known about tomato brown rugose fruit tobamovirus (ToBRFV) regarding longevity in soil and virion stability. Our aims were to determine ToBRFV longevity in naturally-contaminated soil and study virion stability in a range of acidic and alkaline conditions to promote new strategies for soil remediation.

Methods ToBRFV longevity in naturally-contaminated soil was tested by collecting an earth pile after a growth-cycle of ToBRFV-infected tomato plants.

The soil was sampled at different time points and root-truncated tomato seedlings were planted. Virion stability at a range of pH values was determined by testing virus infectivity on *Nicotiana glutinosa*; by amplifying large genome segments using RT-PCR; and by transmission electron microscopy (TEM) visualization.

Results ToBRFV-infectivity in naturally-contaminated soil was profoundly reduced by day 184 of pile-age and was abolished between 205 and 385 days of pile-age. Virion stability and genome integrity were preserved over the pH range of 2–10. At pH 1, ToBRFV-infectivity and efficiency of large genome segment amplifications were reduced. At pH values above 10, modified particle morphologies were visualized by TEM, and virus infectivity was abolished. Treatment of ToBRFV-contaminated soil with an alkaline chlorinated-trisodium phosphate solution profoundly reduced soil-mediated virus infection of root-truncated tomato seedlings.

Conclusions pH values above 10 compromised ToBRFV particle morphology, genome integrity, and virus infectivity. An alkaline disinfectant enhanced soil remediation following natural ToBRFV contamination.

Keywords Soil disinfection · Tobamovirus · pH · Virion stability

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Introduction

Tomato brown rugose fruit virus (ToBRFV) is a positive sense RNA virus belonging to the genus *Tobamovirus*, family *Virgaviridae*. The ToBRFV virion is a rod-shaped particle of approximately 18 nm in diameter and 235 ± 123 nm in length (Luria et al. 2017). The single stranded positive sense RNA genome is about 6400 nucleotides long, encoding the four ORFs of two replicase proteins, the coat protein (CP) and the movement protein (MP) (Luria et al. 2017). Since it was first identified in 2014 in commercial tomato (*Solanum lycopersicum* L.) greenhouses in Israel and Jordan (Luria et al. 2017; Salem et al. 2016), the virus spread to other countries in the Middle East, Europe, and Central and North America, hindering tomato production (Zhang et al. 2022). For decades, the *Tm-2²* resistance allele provided commercial tomato cultivars broad resistance against tobamoviruses (Lanfermeijer et al. 2004; Pelham 1966). However, ToBRFV overcomes *Tm-2²* resistance (Luria et al. 2017). Mutations in MP of tobacco mosaic virus (TMV) and tomato mosaic virus were shown to overcome *Tm-2* and *Tm-2²* resistance alleles, respectively and similarly the resistance breaking of ToBRFV has been recently attributed to the viral MP (Hak and Spiegelman 2021; Meshi et al. 1989; Weber et al. 1993; Yan et al. 2021).

ToBRFV worldwide spread and distribution has prioritized tomato industry research on virus epidemiology, seed transmission, diagnostics and breeding strategies in search of resistant varieties (Zhang et al. 2022; Zinger et al. 2021). Tobamoviruses are highly stable and persist under extreme environmental conditions. Tobamoviruses are very well preserved in plant debris, soil and agricultural tools such as trellising ropes and surfaces in the greenhouse infrastructure and workers' facilities (Broadbent and Fletcher 1963; Broadbent et al. 1965; Lanter et al. 1982). TMV remained infective in buried root debris for at least 13 months (Broadbent et al. 1965; Broadbent 1976). The cucurbit-infecting tobamovirus cucumber green mottle mosaic virus (CGMMV) remained infective in soil for 17 months after the removal of infected watermelon plants (Lovelock et al. 2022). In addition, overwintered soil contaminated with CGMMV-infected plant debris remained infective in bottle gourd propagation (Li et al. 2016). The contaminated soil serves as a primary source of infection

occurring via root-damaged seedlings, which are pre-disposed to soil-mediated viral infection (Broadbent 1965; Dombrovsky et al. 2022).

Tobamovirus persistence in soil depends on various factors including clay content, organic matter, ionic strength and pH (Williamson et al. 2017). Ionic strength and soil pH are negatively correlated with high virus abundance in soil by affecting virus attachment to various surfaces (Gerba 1984; Loveland et al. 1996; Williamson et al. 2017; Yoshimoto et al. 2012). Several soil disinfection strategies such as solarization and steaming, as well as the application of commercially available disinfectants to inactivate tobamoviruses were efficient in reducing virus infectivity (Chanda et al. 2021; Darzi et al. 2020; Dombrovsky et al. 2022; Ling et al. 2022; Luvisi et al. 2015; Panth et al. 2020; Smith and Dombrovsky 2019; Vargas-Mejía et al. 2023). However, mechanisms of disinfectant effects on ToBRFV stability have not been studied yet although a direct effect of pH treatments on viability of the tobamovirus TMV was studied (Best and Samuel 1936; Price 1964; Stanley 1946). The widest pH range found for TMV viable infectious potential was between pH 2 and pH 8 (Best and Samuel 1936).

Regarding soil mediated ToBRFV infection of susceptible plants it was shown that tobamovirus transmission occurs through wounds in root tips, and the virus reaches the upper parts of the plants (Allen 1981; Broadbent 1965; Pares et al. 1992). We have previously shown that ToBRFV was transmitted via soil to susceptible tomato plants under two different study systems. The first was transmission from a viral inoculum poured into the planting pit an hour before planting. The second was transmission from naturally infected soil collected after a growth cycle of an infected crop (Dombrovsky et al. 2022; Klein et al. 2023). In both studies, root-truncated seedlings were planted for the transmission test to increase susceptibility to infection and increase test stringency. We have also found that virus infectious potential in naturally infected soil was apparently dependent on the length of the growth cycle of the infected crop (Klein et al. 2023). However, ToBRFV longevity in soil under the natural contamination conditions has yet not been established.

In the current study, we focused on testing ToBRFV longevity and soil-mediated infection under natural contamination conditions occurring after a 6-month growth cycle of contaminated crops,

and tested infectivity using root-truncated tomato seedlings. In addition, we studied susceptibility of ToBRFV virions to treatments with acidic and alkaline solutions to promote soil remediation strategies that will reduce ToBRFV infectious potential in contaminated soil.

Materials and methods

ToBRFV contaminates soil of commercially grown infected tomato crops

Soil of naturally infected tomato plants was collected after a thorough plant removal establishing a good agricultural practice (GAP) (Fig. 1a) in two commercial sites: Ramat Negev in southern Israel and Ahituv in the Sharon plain in central Israel. The soil was subjected to virion purification and the virome visualized by transmission electron microscopy (TEM) (see below). The purified virions were subjected to large segment (LS) RT-PCR amplifications and a



Fig. 1 Soil collected from commercially grown ToBRFV infected tomato plants and an experimental design for studying ToBRFV longevity in naturally contaminated soil (**a**) A good agricultural practice (GAP): greenhouse workers in Ramat Negev, Israel, remove fruit and plant debris after a growth cycle of contaminated plants before soil sampling. **b** A diagram of experimental design to study ToBRFV longev-

ity in naturally contaminated soil. **c** A pile (kept wet) of naturally contaminated soil collected after a 6-month growth cycle of ToBRFV-infected tomato plants. **d** Sampling at 14 days of a long-term wet earth-pile age. **e** A long-term wet earth-pile, allowing growth of a cover crop. **f** Root truncation of tomato seedlings before planting to test ToBRFV soil-mediated infectivity. **g** Symptoms of severe mosaic in ELISA positive plants

western blot analysis using specific antibodies against ToBRFV (see below) (Luria et al. 2017).

ToBRFV infectivity in soil collected after a growth cycle of virus-infected tomato plants

We have recently demonstrated that ToBRFV in naturally contaminated soil was infectious and infectivity potential was dependent on length of the growth cycle of the infected crop (Klein et al. 2023). For our current study, ToBRFV-infected plants were grown in a commercial soil medium Green 90 (EvenAri, Beit Elazari, Israel) for 6 months to resemble a growth cycle (see experimental design in Fig. 1b). At the end of the growth cycle, the soil was cleared of plant debris excluding the roots; then the soil was collected and piled up. The soil was tested for the presence of ToBRFV by virion purification and a western blot analysis using specific antibodies against ToBRFV (see below) (Luria et al. 2017). Three soil piles were accumulated in a net house: The first was kept dry, the second, a short-term wet pile was irrigated and mixed every 7 days and the third, a long-term wet pile was irrigated twice daily for 20 min and mixed every 15 days, allowing weed cover to grow (Fig. 1c–e). Infectivity potential tests of ToBRFV from the dry earth pile were conducted for 79 days, and from the two wet piles were conducted for 35 and 385 days. Time points for soil sampling from the long-term wet pile were picked in an attempt to extend pile age testing to reach several non-infectious time points. On each testing day, samples from the piles were dispersed into pots and the infectious potential was tested by planting 49 to 57 root-truncated tomato plants cv. Ikram harboring the *Tm-2²* resistance allele. Infectious potential of ToBRFV in soil sampled from the short-term wet earth-pile was tested on 25–27 plants at each time point. Plants were analyzed for ToBRFV infection 30 days post-planting using enzyme linked immunosorbent assay (ELISA) (see below).

ToBRFV virion purifications from soil

ToBRFV virion purification from soil samples was conducted by suspending soil in sodium phosphate buffer (0.1 M, pH=7.0). The suspension was stirred at room temperature for 24 h, and then filtered through Miracloth (Merck, USA). The suspension was then centrifuged for 20 min at 9700 g at 6 °C

using an Avanti J-E centrifuge and a JLA-16.250 rotor (Beckman Coulter, USA). The supernatant was separated and centrifuged for 3 h at 200,000 g at 6 °C using an Optima XPN-90 ultracentrifuge with a Type 45 Ti rotor (Beckman Coulter, USA). The supernatant was discarded, and the pellets were re-suspended overnight at room temperature with 500 µl sodium phosphate buffer (0.01 M, pH=7.0). For further purification and increasing virion concentration, aliquots of 400 µl of the suspended pellet were added to 1.2 ml 20% sucrose in sodium phosphate buffer (0.01 M, pH=7.0). The two-phased solutions were centrifuged for 1 h at 10,000 g at 4 °C. The supernatant was transferred to 5.2 ml vials with the addition of an equal volume of sodium phosphate buffer (0.01 M, pH=7.0), to reduce sucrose concentrations to ~10%, and centrifuged for 3 h at 260,000 g using an SW 55 Ti swinging-bucket rotor (Beckman Coulter, USA). The final supernatant was discarded, and the obtained pellets were re-suspended overnight at 4 °C with 100 µl sodium phosphate buffer (0.01 M, pH=7.0).

Negative staining and transmission electron microscopy (TEM) visualization of ToBRFV

For TEM analysis, soil virion preparations, 2.5 µL of pH-modified ToBRFV virions or Cl-TSP-treated ToBRFV virions (see below) were applied to Formvar/Carbon 300 mesh copper support grids (Ted Pella Inc., USA) for 30 sec. After blotting the excess liquid, a drop of 2.5 µl of 2% (w/v) ammonium molybdate, 0.1% (w/v) trehalose solution (pH=7.2) was applied for 30 sec for negative staining. The remaining excess fluid was blotted, and the grids were allowed to dry for 1 h at room temperature. The samples were visualized using a Tecnai G2 Spirit Twin TEM at 120 kV accelerating voltage (FEI, USA).

Virion RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Virion RNA was extracted from ToBRFV virions using the AccuPrep Viral RNA Extraction Kit (Bioneer, Republic of Korea), according to the manufacturer's instructions. For RT-PCR, viral RNA served as a template for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, USA) with the reverse

primer R-6,392: 5'-TGGGCCCCCTACCGGGGGT TCCG-3' designed for ToBRFV 3' UTR. The cDNA obtained served as a template for PCR amplifications using Platinum™ SuperFi II Green PCR Master Mix (Thermo Fisher Scientific, USA) and specific primers for ToBRFV large segment (LS) PCR amplifications: F-1: 5'-GTGTATTTTTTACAACATATACCA AC-3' and R-6,392 for ~6.3 kb amplicons and F-264: 5'-AGGGCATATCCAGAATTCCA-3' and R-6,332: 5'-ATGTGTATGAACCATACACATTGTC -3' for ~6 kb amplicons. In addition, a ToBRFV specific primer pair (F-5196 5'-GGAGAGAGCGGACGA GGCAA-3' and R-5878 5'-ACAGTTTCCACACT TCGCT-3') was used to obtain a 682 bp segment. Amplicons were Sanger sequenced at the ToBRFV CP region.

Indirect enzyme-linked immunosorbent assay (ELISA)

Tomato leaves were collected, ground in coating buffer (~0.5 g/1 ml), and tested using indirect ELISA (Clark and Adams 1977). Samples were analyzed in duplicates. Laboratory-produced ToBRFV antiserum (Luria et al. 2017), diluted to a ratio of 1:4000 in PBS-milk (2% non-fat milk powder in PBS), was added to the samples. Plates were incubated for 2 h at 37 °C or overnight at 4 °C. Commercial alkaline phosphatase (AP)-conjugated goat anti-rabbit (IgG) antibodies (Sigma-Aldrich, USA, diluted 1:5000 in PBS) were added and incubated for 2 h at 37 °C. p-nitro phenyl phosphate substrate (Sigma) was used at a concentration of 0.6 mg/ml for AP activity detection measured at 405 nm and 620 nm. Samples with optical density (OD) values of 2.5–3 times the negative control were considered ToBRFV positive.

Virion purifications from ToBRFV-infected tomato plants

Tomato plants cv. Ikram were dusted with carborundum and hand-rubbed with a ToBRFV inoculum solution, prepared from infected plant debris homogenized in water. After the emergence of foliar symptoms, 200 g of symptomatic tomato leaves were collected and homogenized in 400 ml sodium phosphate buffer (0.1 M, pH=7.0). Forty ml each of chloroform (stab. Amylene) and *n*-butanol were

added, and the mixture was stirred for 1 h at 4 °C. The mixture was then centrifuged for 20 min at 9700 g at 6 °C using an Avanti J-E centrifuge and a JLA-16.250 rotor (Beckman Coulter, USA). The supernatant was separated and centrifuged for 2.5 h at 200,000 g at 6 °C using an Optima XPN-90 ultracentrifuge and a Type 45 Ti rotor (Beckman Coulter, USA). The supernatant was discarded, and the pellets were re-suspended overnight at 4 °C with 1 ml sodium phosphate buffer (0.01 M, pH=7.0).

pH-modified ToBRFV virions and virion RNA solutions

To prepare pH-modified stock solutions, NaOH (1 M) or HCl (1 M) was added to 0.01 M sodium phosphate buffer with a starting ionic strength of 0.04 M (Fig. S1). To prepare pH-modified virions or virion RNA, 1 µg/µl ToBRFV purified virions or 2 µg/µl virion RNA were diluted 1:10 with each stock solution and incubated for 1 h at 23–25 °C. The final pH of each solution was measured, and no pH deviations were observed. In addition, ToBRFV virions were treated with chlorinated-trisodium phosphate (Cl-TSP) solutions of 1% (pH=11.43), 3% (pH=11.6), 5% (pH=11.6), and 3% Cl-TSP+1% KOH (pH=12.47) as well as NaOH (pH=13), solutions that were used for soil disinfection studies as well (see below).

Biological assays of pH-modified ToBRFV virions and virion RNA solutions

The infectious potential of the pH-treated ToBRFV virions was examined using a biological assay of local lesion (LL) expression on *Nicotiana glutinosa* L. and *Datura stramonium* L. plants. The plants were inoculated with pH-modified virions (~7 µg/leaf) and virion RNA solutions (~5 µg/leaf) to assess quantitatively the effect of pH on ToBRFV infectivity (Luria et al. 2017). Two leaves per plant (1 plant per treatment) were inoculated with each pH-treated virion solution and one leaf per plant (3 plants per treatment using *N. glutinosa* and 2 plants per treatment using *D. stramonium*) was inoculated with each pH-treated virion RNA solution.

Soil disinfection tests of ToBRFV-contaminated soil

To obtain ToBRFV-contaminated soil, ToBRFV-inoculated tomato seedlings were grown in a net house for 4–6 months (see experimental design scheme in Fig. S2). Plant infections were confirmed using ELISA. At the end of the growth cycle, plants were removed, and the soil was collected, mixed, and distributed into five 10 L pots. To test the effect of alkaline pH on ToBRFV-infected soil, four different treatment solutions were prepared, two based on CI-TSP (97% trisodium phosphate, 3% chlorine). Crystalline CI-TSP is a complex structure of sodium hypochlorite (NaOCl), trisodium phosphate (TSP; Na_3PO_4) and water. The CI-TSP complex is stable compared to NaOCl. In aqueous solutions, OCl^- ions are in equilibrium with hypochlorous acid (HOCl), which is unstable. High pH conditions (provided either by NaOH or Na_3PO_4 , or both) shift the equilibrium toward OCl^- and thus inhibit hypochlorite degradation. The four soil treatments were: tap water (pH=8.35), 3% CI-TSP (pH=11.6), 3% CI-TSP+1% KOH (pH=12.47), and tap water with added NaOH (pH=13.0). Each treatment was prepared in 5 L and was poured gradually into the 10 L ToBRFV-contaminated soil pots while the fifth pot served as an untreated positive control. The tap water treatment served as a control for water-mediated virion displacement. The four solutions were incubated with the ToBRFV-contaminated soil for 24 h. Following the treatments, soil was sampled for western blot analyses (see below) and the soil pots were washed three times with 5 L tap water to alleviate the effects of alkaline treatments on soil porosity and structure before testing the soil for ToBRFV infectivity. Remediated soils and the untreated soil were then distributed into 0.3 L pots. Root-truncated tomato seedlings were planted in the pots, 20–25 plants per each treatment, and at 21 days post planting ToBRFV detection was conducted using ELISA. Three experiments were conducted. For statistical analyses, the Two Sample t-Test assuming unequal variances was used.

SDS-PAGE, Coomassie blue staining and western blot analyses

Soil virion preparations were subjected to total protein extractions using the urea-sodium dodecyl sulfate (SDS)- β -mercaptoethanol extraction buffer (75 mM

Tris-HCL pH 6.8, 9 M urea, 4.5% SDS, 7.5% β -mercaptoethanol). The products of the extractions were added to Laemmli loading buffer (Laemmli 1970). In addition, equally sampled pH-modified ToBRFV virions or ToBRFV virions subjected to treatments with the various CI-TSP solutions were added to Laemmli loading buffer. The protein samples were separated on 15% SDS–polyacrylamide gel electrophoresis (PAGE) for 30 min at 40 V followed by 1.5 h at 100 V. Gels were either subjected to Coomassie blue staining or a western blot analysis. For the western blot, gels were electro-blotted onto a nitrocellulose membrane for 30 min at 200 mA using a semidry transfer apparatus (Bio-Rad, California, USA). Membranes were blocked with 3% non-fat dry milk in PBS for 2 h and then incubated with ToBRFV-specific laboratory-produced primary antibodies (1:4000) for 40 min at room temperature and overnight at 4 °C. After four washes with PBS-T (0.05% v/v Tween-20 in PBS), the membranes were incubated with goat anti-rabbit AP-conjugated antibodies (1:5000 in PBS, Sigma-Aldrich, Missouri, USA), followed by four washes with PBS-T. An AP substrate (NBT/BCIP, Bio-Rad, California, USA) was used to detect ToBRFV CP. In the case of CI-TSP treated soil, 24 h after soil treatments with CI-TSP and before soil remediation, 30 g of the treated soils were equally sampled. Ten ml of double distilled water were added to the dry, untreated soil from the control group. The soil mixtures were centrifuged for 15 minutes at 6000 rpm at 6 °C using a Hermle Z 326 K centrifuge and a 221.55 V20 adapter for 50 ml vials (Hermle Labortechnik, Germany). The supernatant was removed, centrifuged again, and added to Laemmli loading buffer and run on SDS PAGE followed by a western blot.

Results

ToBRFV was preserved in soil virome at the end of a commercial growth cycle of ToBRFV-infected tomato plants

Soil samples were collected at the end of a tomato growth cycle in two commercial greenhouses in Israel: Ramat Negev in southern Israel and Ahituv in the Sharon plain of central Israel. The tomato plants were naturally infected with ToBRFV and all aerial plant material was removed (Fig. 1a). Tobamovirus

rod-like particles were apparent in analyses of virion purifications from the soil samples when the soil virome was visualized by TEM, (Fig. 2a–c). Soil ToBRFV was identified by amplifying large genome segments of ~6.3 kb and ~6 kb that were Sanger sequenced at the ToBRFV CP region (Fig. 2d, e). In addition, a western blot analysis using specific antibodies raised against ToBRFV CP (Luria et al. 2017) showed the presence of ToBRFV in the soil (Fig. 2f).

ToBRFV longevity in naturally contaminated soil

For studying ToBRFV longevity in naturally contaminated soil, we piled up soil from a six-month growth cycle of ToBRFV-inoculated tomato plants

to achieve a significant soil-inoculum equivalent to the contaminated soil of a commercial growth cycle. In our model, two different piles were studied: a dry pile, resembling fallowed soil and a wet pile irrigated twice daily to enhance microbial activity and decomposition of organic matter. Virions purified from the accumulated earth pile, tested by a western blot, confirmed ToBRFV contamination of the soil (Fig. 2g). Soil was sampled from the piles at various days of pile-age to test ToBRFV infectivity. For the infectious potential tests, the study was conducted under stringent conditions by performing root truncation prior to seedling planting in order to increase root susceptibility to ToBRFV soil-mediated infection (Fig. 1f). Leaf samples from the planted tomato seedlings of

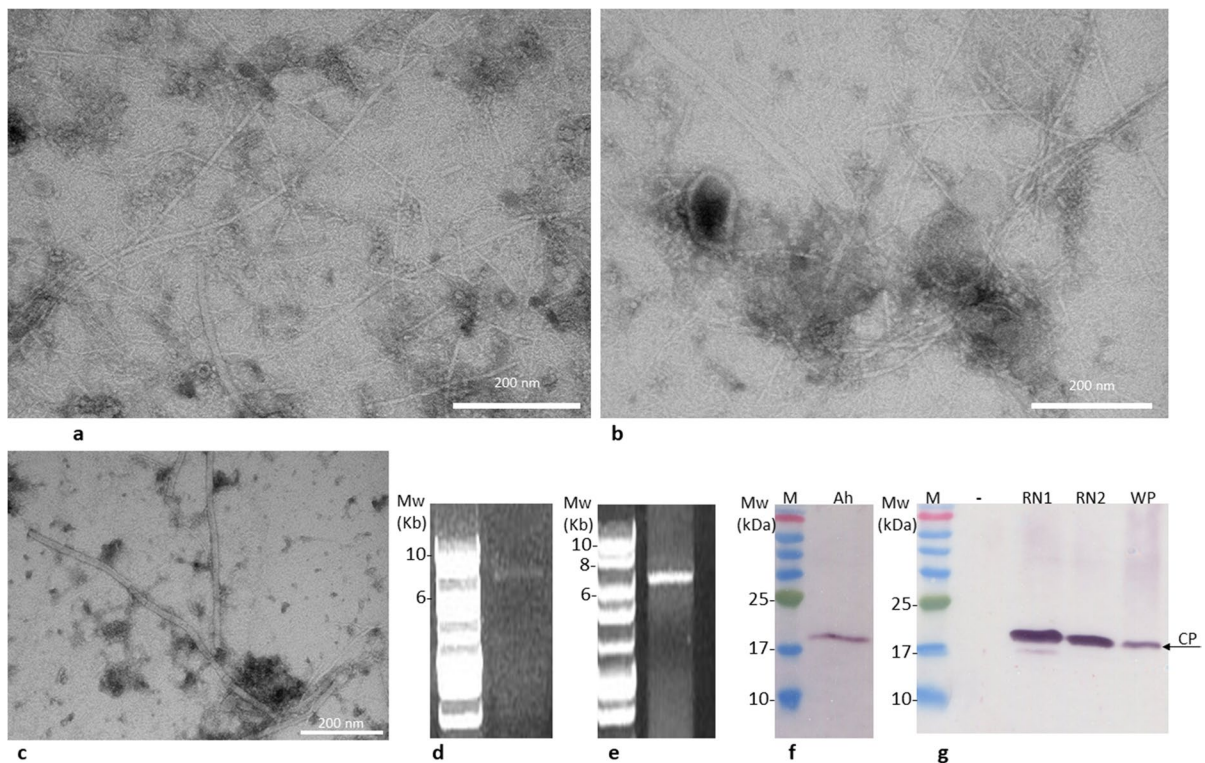


Fig. 2 ToBRFV contaminated soil in commercial greenhouses after a growth cycle of naturally infected crops and following a 6-month growth cycle of virus inoculated plants (**a**, **b**) Representative TEM micrographs of virions purified from soil of a naturally infected crop collected from Achituv, Israel. **c** A representative TEM micrograph of virions purified from soil of a naturally infected crop collected from Ramat Negev, Israel. **d**, **e** LS RT-PCR amplification of ~6.3 kb and ~6 kb large fragments of ToBRFV in soil virions using the primer sets F-1 and R-6392 and F-264 and R-6332, respectively that were Sanger

sequenced at the ToBRFV CP region. **f** A western blot analysis of soil virion purification detected ToBRFV CP of ~17.5 kDa using specific antiserum. **g** A western blot analysis of soil virion purified from Ramat Negev greenhouses and from a wet earth pile accumulated after a 6-month growth cycle. Ah, virions from Achituv greenhouse; RN1 and RN2, virions from two sites in Ramat Negev greenhouses; WP, virions from a wet earth pile (half of the volume was loaded in WP lane compared to RN1 and RN2 lanes); M, molecular size marker

each time-point of pile-age were collected at 30 days post planting and analyzed using ELISA. ToBRFV positive plants were symptomatic (Fig. 1g). ToBRFV longevity was tested for 79 days in the dry earth pile and for 35 and 385 days in the two wet piles (Fig. 3a–c). ToBRFV in the dry earth pile showed a peak of 20% soil-mediated infectivity at the 9th day of pile accumulation, and by the 79th day soil infectivity was reduced to 2% (Fig. 3a). However, ToBRFV in the long-term wet earth-pile showed higher infectivity of 40–50% between 54 and 98 days of pile-age, and reduction to 3% infectivity was observed by day 184 of pile-age (Fig. 3b). These differences could reflect the decomposition of organic matter enhanced in the long-term wet earth-pile that apparently has also assured 0 % infectivity observed between 205 and 385 days of pile-age. A comparison of infectivity at early time points of pile age between the dry and wet earth piles showed similar results manifested in an early peak of infectivity at 15–20 days of the pile age (Fig. 3a, c).

Extreme acidic and alkaline conditions modified ToBRFV virion morphology as visualized by TEM

We asked whether soil pH conditions that affect virus attachment to various surfaces would affect ToBRFV particle stability and infectivity. Purified ToBRFV virions were incubated at different pH conditions for an hour at room temperature (23–25 °C) and were then visualized by TEM (Fig. 4). Rod-like structures characteristic of tobamovirus particle morphology were observed at pH values of 1–10, with a low abundance observed of particles at pH 1 (Fig. 4a–j). At pH 11, truncated rod-shaped virion structures were

apparent of up to ~200 nm in length with abundant discs (Fig. 4k). No distinct structures were observed at pH 12 (Fig. 4l).

Infectious potential of ToBRFV virions and virion RNA was reduced under extreme acidic and alkaline conditions

To find a correlation between virus particle morphology visualized by TEM and ToBRFV infectious potential we performed a biological assay on *N. glutinosa* plants. At pH 1, the average LL counts per leaf was significantly lower than the average LL counts per leaf observed at pH values of 2–10. At pH values of 11 and 12 no LL were observed (Fig. 5a, b). In addition, virion RNA was treated with the various pH solutions and the pH-modified virion RNA was tested for infectivity on *N. glutinosa* plants. pH-treated virion RNA showed no infectivity potential at pH 1 and LL counts were already reduced at pH 10 (Fig. 5a). Because LL counts of pH-modified virion RNA at pH 6 were very low compared to pH 7 when tested on *N. glutinosa* plants, we have retested the infectious potential also on *D. stramonium* plants and found similar infectious potential between the two pH treated RNA samples (Fig. 5a, striped bars).

Genome integrity was compromised at extreme acidic and alkaline conditions

To elaborate on our study of pH effects on ToBRFV virion morphology and infectious potential, we have subjected the viral particles to molecular analyses of ToBRFV genome and coat proteins (see below). ToBRFV virions and RNA extracted from ToBRFV

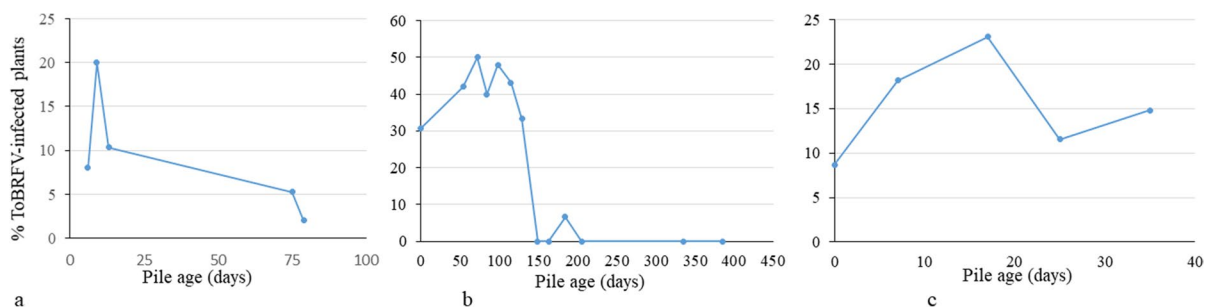


Fig. 3 ToBRFV longevity in naturally contaminated soil collected after a 6-month growth cycle of virus-infected tomato plants **(a)** ToBRFV-infectious potential during 79 days of the

dry earth pile. **b** ToBRFV infectious potential during 385 days of the long-term wet earth-pile. **c** ToBRFV infectious potential during 35 days of the short-term wet earth-pile

virions subjected to pH treatments were analyzed for genome integrity by LS RT-PCR of the ~6.3 kb genome segment (Fig. 6a1, b1). RT-PCR of a ~0.7 kb genome segment was performed as a control for the presence of ToBRFV RNA (Fig. 6a2, b2). LS amplification was readily obtained at pH 3–10 in samples of both the pH-modified ToBRFV virions (in 4–7 reactions of 8) and pH-modified ToBRFV virion RNA (in 3–4 reactions of 4) (Table 1). At pH values above and below that range, LS amplifications were not readily obtained using pH-modified virion samples (in 1–3 reactions of 8) (Table 1). ToBRFV virion RNA showed higher sensitivity to treatments with the extreme acidic and alkaline solutions showing no large genome-fragment amplifications (in four different reactions) at pH values of 1, 2 and 12 (Fig. 6b1, Table 1).

Extreme alkaline pH values promote degradation of ToBRFV coat protein

In addition to particle disassembly at pH 11–12 that was visualized by TEM (Fig. 4k, l), the pH-modified ToBRFV virions were subjected to a western blot analysis and Coomassie blue staining to determine the pH effects on ToBRFV CP. In the western blot, ToBRFV CP was detected using specific antibodies, in all pH-modified ToBRFV virion preparations (Fig. 6c1, d1). However, at pH 12 very low CP levels were apparent. At pH 12, CP degradation products were observed in the Coomassie blue stained gel of the ToBRFV virions (Fig. 6c2, d2). A compound presentation of pH effect on ToBRFV virions showed that a positive correlation generally occurred between abundance of rod like particles, local lesion manifestations and readily obtained LS amplification of RNA from pH treated virions (Fig. 6e). However, pH 2-treated virions did not show a clear positive correlation between LS amplification ratios (3 out of 8 reactions, Table 1) and the obtained high LL count (Fig. 5a).

Soil treatment with alkaline solutions reduced ToBRFV soil-mediated infectious potential

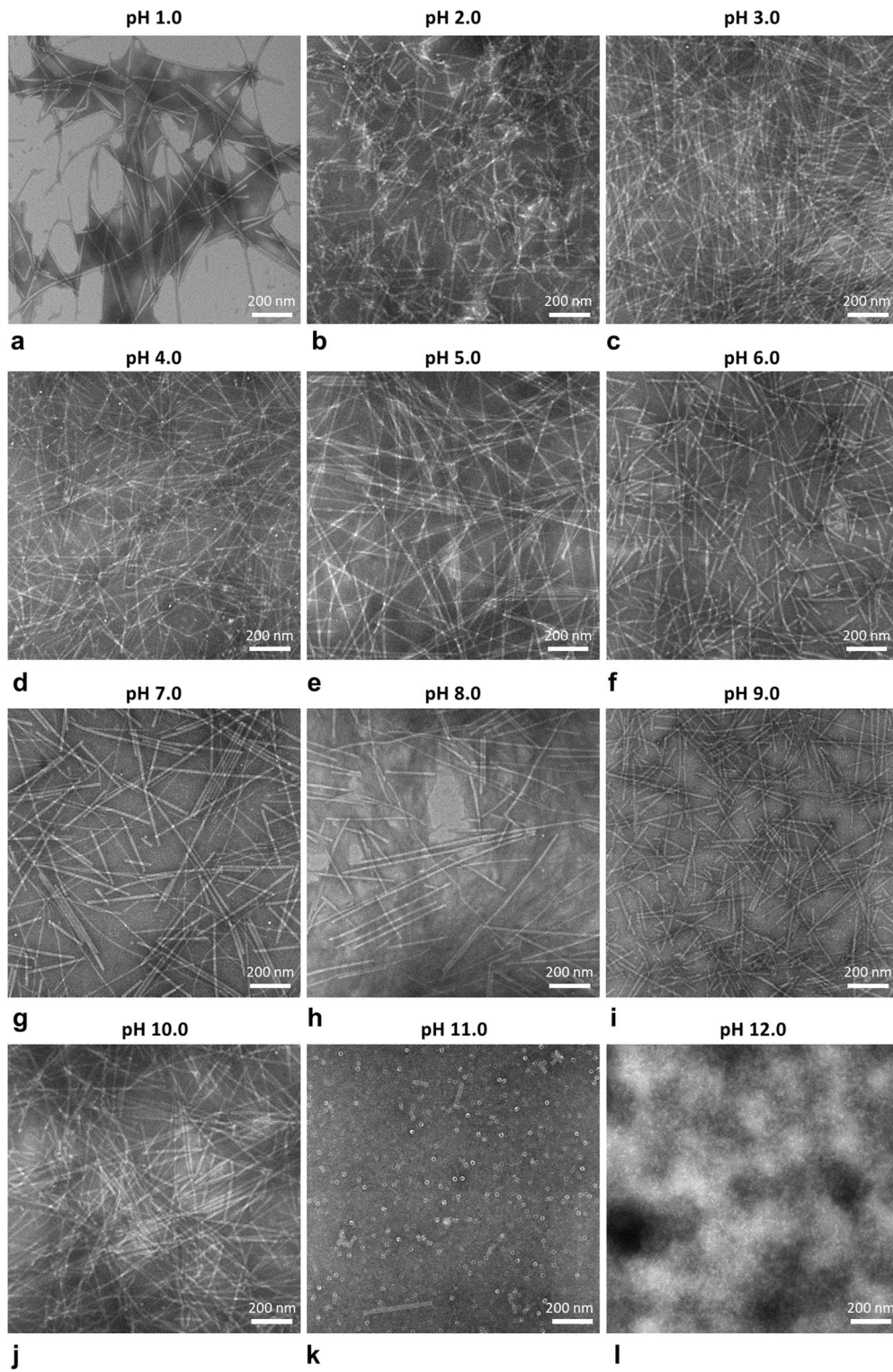
To exploit the high pH effects on ToBRFV particles, genome integrity and infectious potential we have assayed a soil disinfection strategy employing CI-TSP solution for disinfection of the naturally contaminated

soil. Soil accumulated after a 4–6 month growth cycle of ToBRFV infected tomato plants was used for the assay (Fig. S2). Soil treatment with either 3% CI-TSP solution (pH 11.6) or with 3% CI-TSP+1% KOH (pH 12.47) showed a significant reduction in infectious potential by soil-mediated ToBRFV transmission, compared to untreated control ($p < 0.05$; Two Sample t-Test assuming unequal variances) (Fig. 7a). Tomato plants grown in soil incubated with 3% CI-TSP or 3% CI-TSP+1% KOH showed the low infectivity of $6.3 \pm 6.3\%$ and $5 \pm 5\%$, respectively, compared to the untreated control (infectivity of $39.7\% \pm 9.0$). Tomato plants grown in soil incubated with tap water (pH 8.35) showed $19.1 \pm 17.7\%$ infectivity. The high variability in the results of tap water effect hampers any prediction regarding the alkaline water efficiency as a soil disinfectant. Treatment with NaOH (pH 13) apparently caused ToBRFV degradation; however, soil structure was modified showing dispersion that led to plant mortality (Fig. 7a). The 3% CI-TSP effect on soil disinfection was confirmed by testing ToBRFV CP content in the treated soil of one experiment using a western blot analysis (Fig. 7b). Tap water treatment in that experiment showed reduction in ToBRFV CP levels as well. The effect of CI-TSP solutions on ToBRFV purified virions was also examined by TEM visualization and Coomassie blue staining following SDS-PAGE. Virion particles could not be visualized by TEM and virion CP could not be detected by the Coomassie blue-stained gel (Fig. 7d–f, g).

Discussion

In our study, we aimed to investigate perseverance of ToBRFV infectious potential in naturally contaminated soil and to characterize virion stability under acidic and alkaline conditions with respect to particle morphology, infectivity and genome integrity. Our results could be used to improve various soil disinfection protocols that will contribute to GAP and we have applied our conclusions in a suggested protocol using alkaline CI-TSP solutions.

For ToBRFV longevity studies, two types of earth piles kept either dry or wet were tested for ToBRFV infectious potential. The long-term wet pile apparently increased decomposition of organic matter and we observed an increase in ToBRFV



◀**Fig. 4** Representative TEM micrographs of pH-modified ToBRFV virions. **a–j** Rod like particles characteristic of tobamoviruses were visualized in pH-modified virions over the pH range 1–10. **k** Truncated virions of up to ~200 nm and structures resembling 20S discs were visualized in virion solutions incubated at pH 11. **l** Undefined structures were observed in virion solutions incubated at pH 12

infectious potential until the 129th day of pile assembly, whereas the dry earth pile showed reduction to 2% infectious potential on the 79th day of pile-age. The decomposition of organic matter in the wet pile apparently allowed the elimination of ToBRFV infectivity by day 205 of pile-age (Fig. 3).

Our study of pH effects on ToBRFV particle stability showed that infectivity potential of ToBRFV was stable between the pH values of 2–10 tested on *N. glutinosa* plants (Fig. 5). These results differ from the results describing TMV susceptibility to pH treatments that showed particle stability over the pH range of 2–8 (Best and Samuel 1936), or the results describing TMV rapid inactivation below pH 3 and above pH 8 (Price 1964; Stanley 1946). However, a low pH 1 reduced infectivity potential of both TMV and ToBRFV tested on *N. glutinosa* plants (Fig. 5). At pH 1, TEM visualization of ToBRFV displayed reduced levels of rod-like particles and the amplification of a large genome segment by LS RT-PCR was not readily obtained (Fig. 4a, Table 1). Importantly, at the high pH 9, TMV was partially inactivated, with the greatest fall in activity occurring in the first few minutes (Best and Samuel 1936), implying that differences in incubation-time could not explain differences between TMV results and ToBRFV. At pH 9 and 10, TEM visualization of the pH-treated ToBRFV virions showed high concentrations of rod-like particles (Fig. 4i, j). In addition, the infectious potential of ToBRFV using *N. glutinosa* plants was not significantly different from that occurring at pH 7 and a large ToBRFV genome segment was readily amplified by LS RT-PCR (Figs. 5 and 6a1, Table 1). These results also differ from studies of TMV CP disassembly that showed reduced bond strength at pH 9 (Caspar 1964).

At the high pH 11, ToBRFV infectivity was lost, as tested on *N. glutinosa* plants, and TEM visualization showed truncated rod-like particles of up to ~200 nm in length, along with discs resembling 20S discs formed by TMV CP at the low pH values of 6.5–7 (Figs. 4k and 5) (Kegel and Van Der Schoot

2006; Klug 1999). As the pH is lowered, there was an increase in TMV CP rod length and at higher pH values, 4S aggregates were apparent (Bhyravbhatla et al. 1998). These data suggest that keeping the high pH values of tobamovirus solutions would prevent reformation of rod-like particles. In addition, studies of the TMV intracellular disassembly process in which carboxylate interactions were assumed to play a role, revealed that in alkaline solutions and low calcium ion concentrations, destabilization of the interaction between adjacent capsid subunits leads to viral disassembly (Caspar 1964; Weis et al. 2019). At pH 12 no defined structures were visualized by TEM of ToBRFV virions and the ToBRFV CP was degraded (Figs. 4l and 6d), which could prevent any potential reassembly.

In our study, we have observed a tight positive correlation between infectious potential and LS genome amplification of pH treated ToBRFV virions at the pH range of 3–10 (Fig. 6e). Although it seemed redundant, we have also analyzed susceptibility of ToBRFV extracted virion RNA to pH treatment and tested the infectious potential as well as LS genome amplification. Apparently, when comparing treatments of virion RNA with pH 3 and pH 10, LS amplification was similar between the treatments (Table 1) but infectious potential was significantly reduced at pH 10 (Fig. 5), suggesting that the naked RNA secondary structures were highly susceptible to the destructive effects of alkaline pH values compared to acidic pH values.

For soil disinfection strategies, it is necessary to take into consideration virus attachment to various surfaces in the aquifer sediments, primarily controlled by electrostatic interactions (Loveland et al. 1996). The pH at the isoelectric point (pH_{iep}) of the tobamovirus TMV was determined as ~3.5 (Eriksson-Quensel and Svedberg 1936). Accordingly, in solutions with pH values below 3.5 we could expect the tobamoviruses to be positively charged and most aquifer grain surfaces are positively charged below pH 4 (Loveland et al. 1996). However, we could not benefit from this repulsion between the tobamoviruses and most soil grain surfaces because at the acidic pH 1 there was a partial infectivity of ToBRFV observed on *N. glutinosa* plants and at pH 2 ToBRFV was highly infective (Fig. 5). Over the pH range 4–9 most aquifer grain surfaces are negatively charged (Loveland et al. 1996) and the tobamovirus would be negatively charged

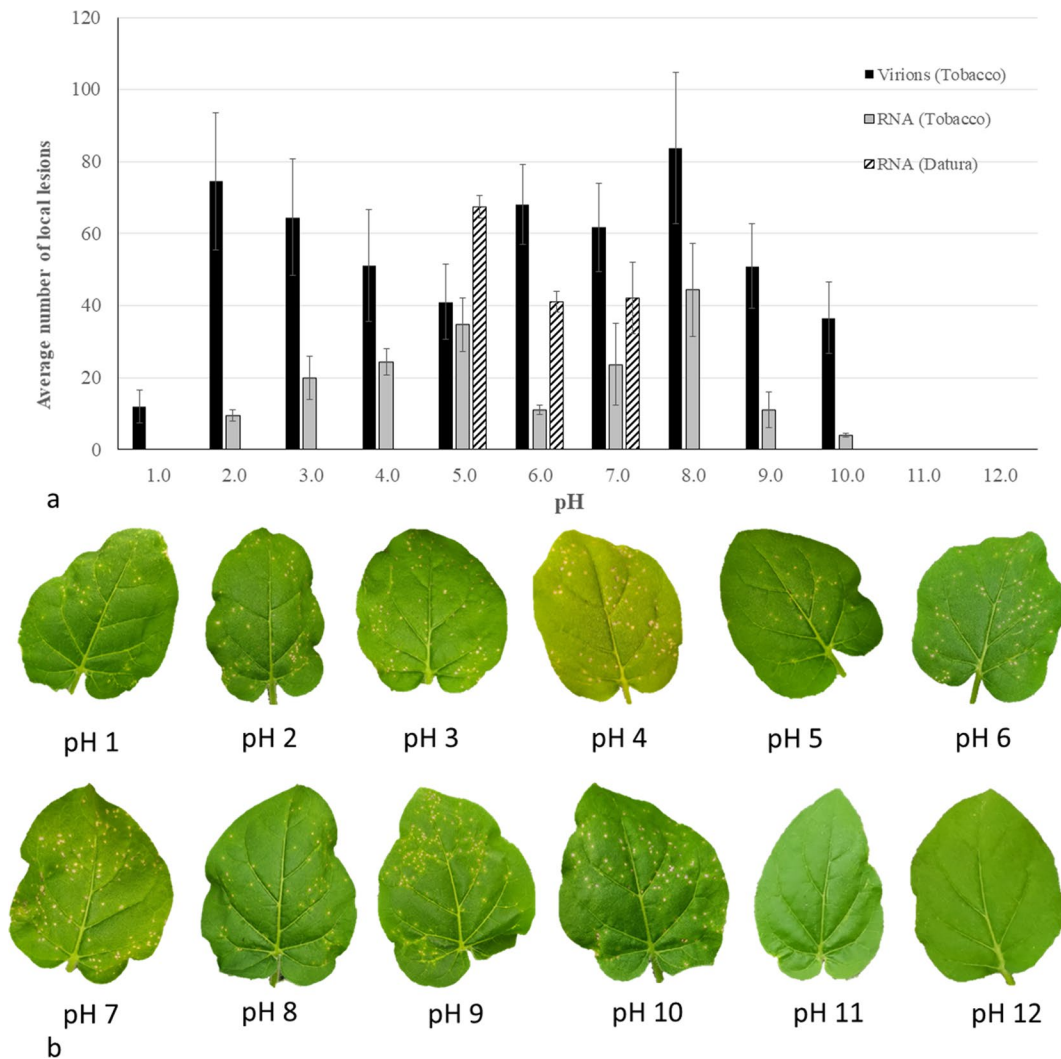


Fig. 5 Extreme acidic and alkaline solutions reduced the infectious potential of ToBRFV virions and virion RNA. **a** Local lesion (LL) counts of pH treated virions and virion

RNA using *N. glutinosa* and *D. stramonium* plants. Bars represent average LL count $\pm$ SEM. **b** Sampled *N. glutinosa* leaves showing local lesions of pH-modified virions

as well. However, the results of soil incubation with water at pH 8.35 showed low consistency of infectivity, suggesting that the electrostatic repulsion was not strong enough to promote ToBRFV displacement from soil.

To obtain soil treatment at pH ~11 that compromised ToBRFV genome integrity and eliminated virion infectivity tested on *N. glutinosa* plants (Figs. 5 and 6, Table 1) we used a 3% CI-TSP solution (pH 11.6). In previous studies, soil treatment with 3% CI-TSP reduced ToBRFV infectivity by inactivating a soil inoculum of ToBRFV (Dombrovsky

et al. 2022; Klein et al. 2023). We now found that treatment of naturally contaminated soil with 3% CI-TSP significantly reduced soil-mediated infection of tomato plants compared to the untreated positive control concomitant with reduction in ToBRFV CP levels (Fig. 7a, b). In a study of TSP activity on disruption of bacterial cell membranes the TSP effect was attributed to the solution pH values (Sampathkumar et al. 2003). However, our analyses of direct effect of CI-TSP solutions at pH 11.6 on virion integrity visualized by TEM and Coomassie blue stained gel showed that virions and CP were undetectable, an effect more

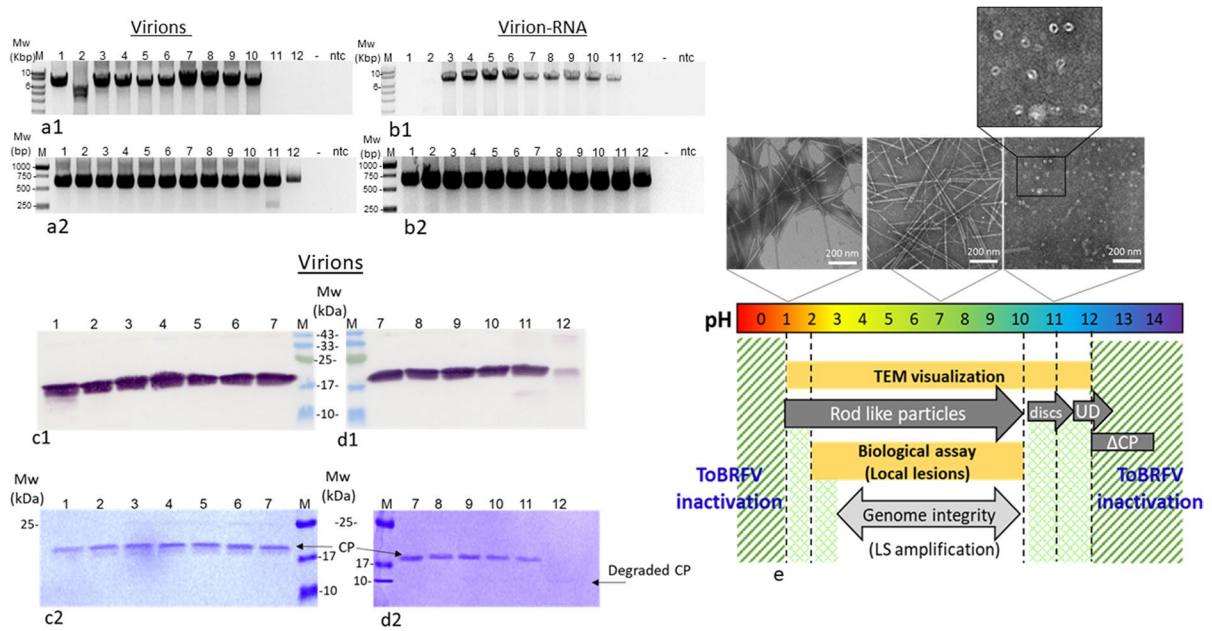


Fig. 6 ToBRFV compromised genome integrity and CP degradation occurred under extreme alkaline conditions and positively correlated with reduced infectious potential and modified particle morphology. (a1) pH-modified virions subjected to LS RT-PCR amplifications of a large ~6.3 kb fragment using the primer pair F-1 and R-6392. (a2) pH-modified virions subjected to RT-PCR amplifications of a small ~0.7 kb fragment using the primer pair F-5196 and R-5878. (b1) pH-modified virion RNA subjected to LS RT-PCR amplifications of a large ~6.3 kb fragment. (b2) pH-modified virion RNA subjected to RT-PCR amplifications of a small ~0.7 kb

fragment. (c1, d1) Western blot analyses of pH-modified virions showing the ~17.5 kDa CP. (c2, d2) Coomassie blue-stained SDS-PAGE gels of pH-modified virions showing the ~17.5 kDa CP and degradation products at pH 12. Numbers at the top of the figures are the treatment pH; ntc, negative template control; M, molecular size marker. (e) A compound figure showing a positive correlation between the compromised genome integrity at high pH values, the modified particle morphology and the abolished infectious potential. UD, undefined structures; ΔCP, degraded CP

Table 1 pH effect on large segment amplifications using ToBRFV virions or virion RNA templates

pH	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0
Virions												
*LS-PCR amplifications/total assays	2/7	3/8	7/8	7/8	7/8	6/8	6/8	5/8	7/8	4/8	1/8	1/8
Virion RNA												
LS-PCR amplifications/total assays	0/4	0/4	3/4	4/4	4/4	3/4	4/4	4/4	4/4	3/4	1/4	0/4

*LS large segment

severe than the effect of the high pH 12 on ToBRFV virions (Figs. 4 and 7). Therefore, a triple mechanism of disinfection is suggested to be attributed to Cl-TSP solutions: (i) the phosphate acts as a surfactant; (ii) the high pH causes protein degradation; (iii) the

hypochlorite in aqueous solutions acts as an oxidizing and chlorinating agent.

To conclude: our data demonstrated that ToBRFV contaminated the soil following a growth cycle of naturally infected crops and the virus was highly

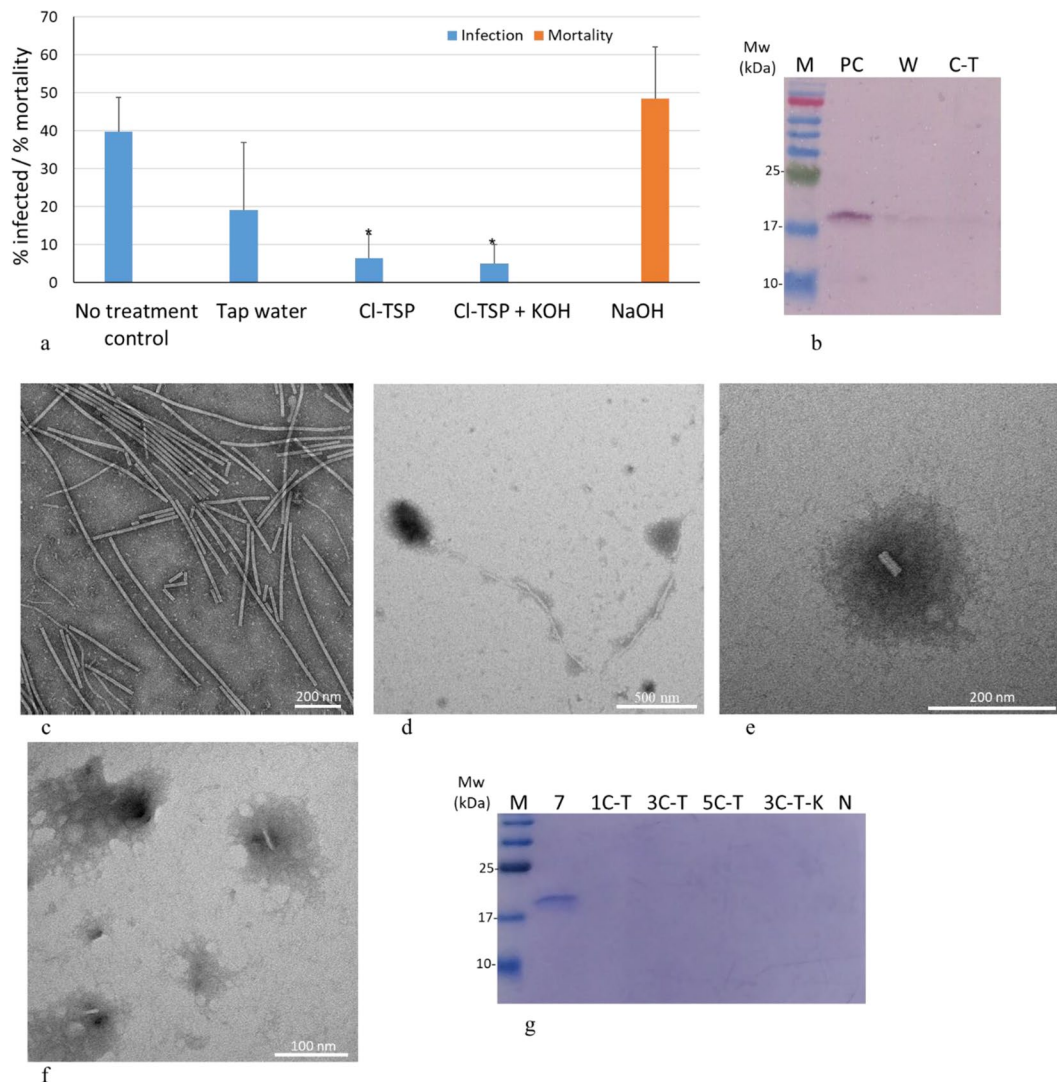


Fig. 7 Soil disinfection with alkaline CI-TSP solutions reduced ToBRFV soil-mediated infection of tomato seedlings and caused disintegration of ToBRFV virions. **a** ToBRFV soil-mediated infection of tomato seedlings following soil disinfection treatments with tap water pH 8.35 ($n=78$), 3% CI-TSP pH 11.6 ($n=78$), 3% CI-TSP+1% KOH pH 12.47 ($n=86$) and NaOH pH 13 ($n=81$). Infectivity and mortality of tomato plants are presented as $\% \pm$ SEM. Untreated soil served as a positive control ($n=108$). Asterisks represent a significant reduction $p < 0.05$ (Two Sample t-Test assuming unequal variances) compared to the untreated positive control. **b** A western blot analysis of ToBRFV CP in 3% CI-TSP treated soil. PC, positive control; W, tap water; C-T, 3% CI-TSP. **c** A representative TEM micrograph of ToBRFV virions at pH 7 (**d**) A representative TEM micrograph of ToBRFV virions treated with 1% CI-TCP. **e** A representative TEM micrograph of ToBRFV virions treated with 3% CI-TCP; **f** A representative TEM micrograph of ToBRFV virions treated with 3% CI-TCP+1% KOH. **g** A Coomassie blue stained gel of ToBRFV virions treated with soil disinfection solutions. 7, pH 7; 1C-T, 1% CI-TSP pH 11.43; 3C-T, 3% CI-TSP pH 11.6; 5C-T, 5% CI-TSP pH 11.6; 3C-T-K, 3% CI-TSP+1% KOH pH 12.47; N, NaOH pH 13; M, protein molecular size marker

stable and infectious at the pH range of natural waters (pH 4–9) (Loveland et al. 1996). To prevent soil-mediated ToBRFV infection, enhancement of soil disinfection could be provided by the use of alkaline disinfectant solutions or addition of alkaline solutions

to other disinfection strategies such as steaming (Luvisi et al. 2015) or solarization.

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Data availability All data is presented in the manuscript.

Declarations

Competing interests The authors declare no conflict of interest.

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