Detection of tomato brown rugose fruit virus in environmental residues: the importance of contextualizing test results

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Abstract

Tomato brown rugose fruit virus (ToBRFV) is regulated as a quarantine pest in many countries worldwide. To assess whether ToBRFV is present in cultivations, plants or seed lots, testing is required. The interpretation of test results, however, can be challenging. Real-time RT-PCR results, even though considered “positive”, may not always signify plant infection or indicate the presence of infectious virus, but could be due to the presence of viral residues in the environment. Here, case studies from the Netherlands, Belgium, and the United Kingdom address questions regarding the detection of ToBRFV in various settings, and the infectiousness of ToBRFV positive samples. These exploratory analyses demonstrate widespread detection of ToBRFV in diverse samples and environments. ToBRFV was detected inside and around greenhouses with no prior history of ToBRFV infection, on different materials and surfaces including those that were untouched by individuals, plants, or objects. This suggested the dispersal of viral residues through aerosols. ToBRFV or its residues were more often detected in areas with nearby tomato production yet were also found in a wider environment extending beyond infected crops. Given that ToBRFV originating from environmental contamination may or may not be infectious, adds complexity to decision-making in response to positive test results. Contextual information, such as the origin of the sample and the likelihood of residues from prior cultivations and/or the broader environment, is important for interpreting test results. A nuanced approach is crucial to correctly interpret ToBRFV test results, necessitating further research to support risk assessment.

Introduction

Tomato brown rugose fruit virus (ToBRFV) is a tobamovirus that may cause severe disease in tomato and pepper crops. Therefore, it is regulated as quarantine pest in numerous countries, necessitating testing to establish its presence or absence (EPPO, 2024). ToBRFV was initially identified in 2015 in Jordan (Salem *et al.*, 2016), and has currently been reported from Europe, Africa, North America, South America and Asia (EPPO, 2024). Natural hosts of ToBRFV include tomato (*Solanum lycopersicum*) and pepper (*Capsicum* spp.) (Luria *et al.*, 2017, Panno *et al.*, 2020, Salem *et al.*, 2020). Infected tomato plants may show mosaic patterns on leaves, narrowing of leaves, and the appearance of yellow spots or brown wrinkled (rugose) symptoms on fruits (Luria et al., 2017, Salem et al., 2016). ToBRFV poses a significant threat to tomato production, given that the established tobamovirus resistance genes in tomatoes, namely *Tm-1*, *Tm-2* and $Tm\text{-}2^2$, are ineffective against this virus (Luria et al., 2017). For pepper, reported symptoms encompass yellowing, necrotic lesions or spots, and discoloration of fruits. The resistance provided by the L^3 and L^4 genes or alleles may break down at temperatures exceeding 30°C (Eldan *et al.*, 2022, Fidan *et al.*, 2021).

Like other tobamoviruses, ToBRFV is highly stable and easily transmitted through contact and propagation materials. Furthermore, ToBRFV is seedborne and seed-transmitted, as it can be located externally on the seed coat (Davino *et al.*, 2020, Salem *et al.*, 2022b). Several territories have listed ToBRFV as a quarantine pest and the European Union (EU) has implemented emergency measures since 2019 for tomato and pepper (EU, 2019), to prevent the entry and spread of the virus. Phytosanitary controls within these emergency measures include statutory surveillance through testing by (real-time) RT-PCR. In addition, appropriate measures to eradicate ToBRFV from infected production sites are required by the end of the cropping cycle.

However, it has been hypothesized that positive test results do not always indicate plant infection but could be attributed to the presence of residual virus in the environment, for instance originating from highly infected plants in close proximity (Skelton *et al.*, 2023b). This raises questions regarding how to correctly interpret positive test results and the potential risks associated with a broader presence of the virus in the environment. The virus's persistent nature, the ease of transmission through contact and presumed environmental spread coupled with highly sensitive diagnostic tests, make interpreting positive test results complex. It is particularly challenging to discern whether plants are genuinely infected or if the detected presence of ToBRFV is due to environmental contamination.

Assessment of the phytosanitary risk posed by environmental residues adds another layer of complexity. There has been limited work related to the phytosanitary relevance and transmission risk of environmental ToBRFV. One study indicated that irrigation with ToBRFV-contaminated water can lead to plant infection (Mehle *et al.*, 2023). Another study suggested that the presence of ToBRFV residues in a greenhouse could lead to crop infection. The latter hypothesis was supported by the identification of almost identical

ToBRFV sequences in a tomato crop during a ToBRFV outbreak and in the subsequent crop in the same greenhouse several months after disinfection and crop rotation (van de Vossenberg *et al.*, 2021). Apart from the transmission risk, ToBRFV in environmental residues also poses a 'diagnostic risk' of erroneously reporting crop infection in the absence of truly infected plants (Skelton et al., 2023b).

To gain more insight into the detection of ToBRFV in various environmental settings and its potential phytosanitary implications, case studies were conducted in the Netherlands, Belgium, and the United Kingdom (UK). Collectively, these studies focused on the following questions:

1. Can ToBRFV be detected on presumed nonhost plants and inanimate surfaces, not in direct contact with infected plants, individuals, or objects, suggesting environmental contamination (ToBRFV residues)? (case study 1, 2, 3, 5)
2. Can ToBRFV residue be infectious? (case study 1, 3, 4, 6)
3. Is there a distinction between locations with and without prior ToBRFV outbreaks? (case study 1)
4. Are there differences between regions with high and low concentrations of (infected) tomato production? (case study 1)

These exploratory analyses demonstrate the presence of ToBRFV residues in diverse settings, even in the absence of direct contact with infection sources, thereby offering insights into the potential implications caused by the presence of environmental residues.

Materials and Methods

Case study 1 (Netherlands)

Various samples, including leaves, swabs from a range of surfaces, water and soil or substrate, were collected in and around greenhouses in the Netherlands producing either cucumber (*Cucumis sativus*) or green bean (*Phaseolus vulgaris*), which are nonhosts of ToBRFV. Some of the greenhouses had grown tomato and experienced a ToBRFV outbreak in the past half year before sampling, but after crop cleanout and disinfection they had switched to the production of a nonhost crop in all or part of their compartments. The geographic regions where sampling was carried out either had a high or a low concentration of tomato production in the West of the Netherlands (sampled in June-July 2023) and the East of the Netherlands (sampled in October 2023) respectively (Fig 1a). The choice of samples per location differed slightly (Table 1), due to the exploratory nature of this study and the unique characteristics of each location. The primary sample types consisted of swabs and leaves. Swabs were collected from surfaces that were either typically untouched (e.g. ceiling structures) or had been exposed to contact with individuals, plants or objects. For both cucumber and green bean, lower leaves were sampled, along with dead leaves from the floor. For tomato, young leaves from the top of the plants were bulk sampled. Various additional samples included soil from beneath the plastic covers, rockwool, paperwork,

volunteer tomato plants (company 6 only), wastewater, rainwater, and disinfected water. Outside greenhouses, leaf and swab samples were collected from various plants and different surfaces.

Case study 2 (Netherlands)

Leaf or sepal samples of tomato (*S. lycopersicum*), drain water and swab samples from different surfaces with varying levels of contact with individuals, plants, or objects, were collected at three adjoining tomato production greenhouses belonging to the same company in the West of the Netherlands (Fig 1a). To our knowledge, none of the sampled locations had been previously infected with ToBRFV. Samples were collected in the summer of 2021.

Case study 3 (Belgium)

Weed samples were collected within 3 m outside of a tomato production greenhouse situated in an area with a high concentration of tomato production in the North of Belgium in the summer of 2023 (Fig 1b). This company likely experienced a ToBRFV outbreak at the time of sampling, as the National Plant Protection Organization had been notified of an infection earlier in the season. The following weed species, common to Western Europe, were sampled randomly: *Erigeron canadensis, Galinsoga quadriradiata, Solanum nigrum,* and *Sonchus oleraceus*.

Case study 4 (Belgium)

High throughput genome sequencing (HTS) analysis was performed on two tomato leaf samples collected in 2020-2021 from two adjoining tomato production greenhouses of the same company situated in an area with a high concentration of tomato production in the North of Belgium (Fig 1b). The crops at both locations became infected with ToBRFV shortly after each other.

Case study 5 (United Kingdom)

In February 2022, swab samples were collected from different surfaces in a tomato production facility consisting of two conjoined greenhouses, in the West Midlands, UK (Fig 1c) with an ongoing ToBRFV outbreak, first detected in March 2020. Many of the sampled surfaces had not been in direct contact with individuals, plants, or objects, e.g., the greenhouse roof structure (inside), tops of roof lights, and inside fans. Other surfaces included bee boxes, the underside of the gutters, a Tyvek suit, and the grower's phone and gloves. The Tyvek suit was sampled immediately after it was removed by the person taking the swab samples. The sampled area specifically targeted the section where the person had leaned against a retaining bar on a scissor lift, for support, whilst taking a swab sample from the greenhouse roof. The surrounding area harboured a low concentration of tomato production.

Case study 6 (United Kingdom)

In March 2023, swab samples were collected from different surfaces of a ToBRFV experimental greenhouse in the North-East of England, UK (Fig 1c) where several ToBRFV

155 inoculation experiments had taken place, including a ToBRFV-tomato sampling trial (Skelton
156 et al, 2023b), followed by smaller ToBRFV experiments with infected plants. After the initial
157 tomato trials, the greenhouse had been disinfected several times with Virkon (SLS Ltd, UK).
158 The greenhouse had not contained ToBRFV-infected plants for at least one month before
159 sampling. Swab samples were taken pre and post treatment with a hydrogen peroxide-based
160 fogging product (BioDecon, UK). The surrounding area harboured a low concentration of
161 tomato production, with the nearest producer situated over 40 km away.

162 ## Sampling details

163 Case study 1: The cotton bud of a sterile swab was moistened by dipping it into a 1.5 mL
164 tube with 150 µL DNAse/RNAse free water. The moistened bud was used to swab
165 approximately 5 times across the target surface for a length of 5-10 cm while slightly rotating
166 the stick after each swab. The bud was stored in the same 1.5 mL tube by snapping off the
167 stick. A leaf sample consisted of leaf pieces of approximately 1-2 cm^2 from 10 plants of the
168 same species, or from an equivalent number of different leaves if less plants were present.
169 Other samples included a piece of soil of approximately 1 cm^3 collected from below the
170 plastic covers on the greenhouse floor, a similarly sized piece of rockwool used for plant
171 cultivation, or an approximately 2 cm^2 piece of administration paper. Approximately 5 mL of
172 wastewater, rainwater, or disinfected water was collected in tubes. Samples were collected
173 in biological duplicates, by taking two swabs at the same time or dividing collected material
174 in two equal parts or volumes.

175 Case study 2: Swab samples were taken by wetting the white cotton head of the swab
176 (EUROTUBO® Collection swab, deltalab) in 1 mL sterile PBS (pH 7.4) and moving it back and
177 forth over the surface of interest. Leaf samples were taken from 10 different tomato plants
178 in 10 different rows spread randomly throughout the greenhouse for each location.
179 Greenhouse drain water was sampled from a collection reservoir awaiting disinfection, as
180 described by Mehle et al. (2023).

181 Case study 3: Each sample consisted of leaf material from one plant per species.

182 Case study 4: Tomato leaf samples were taken as described for case study 2. At the time of
183 sampling the tomato plants at location 1 were close to the end of the cropping cycle,
184 whereas the plants at location 2 were younger, closer to the beginning of the cropping cycle.

185 Case study 5: Swab samples were taken by soaking cotton buds in 1 mL of 0.06 M phosphate
186 buffer (pH 7) in a 50 mL universal tube. The soaked cotton bud was then used to swab
187 approximately 20 cm^2 of target surface, returned to the universal tube and soaked for at
188 least 30 minutes. In addition, symptomatic leaf material, approximately 4 cm^2, was taken
189 from the growing crop.

190 Case study 6: Swab samples were taken from different greenhouse surfaces (door handle,
191 glass pane, concrete floor, steel bench, aluminium ladder and watering lance), both pre and

post treatment, with a hydrogen peroxide-based fog product at 7.5% active ingredient (approximately 40 min treatment).

In all case studies care was taken to avoid cross contamination and gloves were changed when taking different samples. Samples were stored at -20°C (case study 1) or 4°C (case studies 2-6) until RNA extraction.

RNA extraction and (real-time) RT-PCR

Case study 1: 700 µL of guanidine hydrochloride (GH+) buffer (EPPO, 2022) was added to each swab sample and vortexed for 15 sec. Leaf, paper, soil, and rockwool samples were homogenized with GH+ buffer at an approximate ratio of 1:2-1:5 [w/v]. Each water sample was mixed with GH+ buffer at a ratio of 1:10 [v/v]. RNA was extracted from 500 µL of homogenate using the Qiagen RNeasy Plant Mini kit (manufacturer's instructions). Real-time RT-PCR for ToBRFV was performed based on the protocol of the International Seed Health Initiative for Vegetable Seeds using the CaTa28 and CSP1352 primers and probes (ISF, 2020, EPPO, 2022) with two modifications: the TaqMan RNA-to-CT 1-step Kit (Applied Biosystems) was used instead of the Ultraplex 1-step Toughmix (Quantabio) and 2 µl of total RNA was used instead of 5 µl. The plant gene NADH dehydrogenase subunit 5 (nad5) was used as an internal control for RNA extraction from leaf samples (Botermans *et al.*, 2013). Additional negative controls included cucumber and tomato leaves from young non-infected plants and clean printer paper.

Case study 2: RNA was extracted from 100 mg leaf or sepal tissue or from 300 µL of swab samples in PBS, using the RNeasy Plus kit (Qiagen). Swab samples were first thoroughly vortexed to stimulate release of the viral particles. Samples were analyzed by real-time RT-PCR for ToBRFV using the CaTa28 and CSP1352 primers and probes (ISF, 2020, EPPO, 2022)

Case study 3: RNA was extracted from leaf samples using the KingFisher Apex system (ThermoFisher) with the MagMAX™ Plant RNA isolation kit (ThermoFisher). Samples were further analyzed by real-time RT-PCR for ToBRFV using the CaTa28 and CSP1352 primers and probes (ISF, 2020, EPPO, 2022). Additionally, RNA of the *S. oleraceus* and *G. quadriradiata* samples was used to perform a ToBRFV-specific near-full genome PCR (hereafter referred to as NFG-PCR) to determine the integrity of the detected RNA (Mehle et al., 2023), using primers F-22 and R-6392 as previously described by Eldan et al. (2022).

Case study 4: Samples were extracted and analyzed as described for leaf samples in case study 2 to confirm infection with ToBRFV.

Case Study 5 and 6: RNA was extracted from the swab buffer using the Qiagen RNeasy plant mini kit (manufacturer's instructions). Nucleic acid was extracted from leaf material using GH+ buffer at 1:10 dilution. Leaves were ground using a HOMEX 6 (Bioreba) and RNA was extracted by magnetic bead extraction using Invimag Virus DNA/RNA mini-kit (Invitek GmbH). RNA extracts were tested by real-time RT-PCR with the Menzel and Winter primers (Menzel & Winter, 2021, EPPO, 2022) using iTaq universal probes one-step reaction mix (Bio-

230 Rad) and 1 µl of RNA extract. Samples were tested in duplicate and Cq-values recorded.
231 Cytochrome oxidase (COX) was used as an internal control for RNA extraction from leaf
232 samples (Weller *et al.*, 2000).

233 Appropriate positive and negative (extraction) controls were used in all case studies and
234 performed as expected. Samples were considered positive when amplification curves were
235 observed.

236 Bioassay

237 Case study 1: Mechanical inoculations and cultivation of test plants were performed as
238 described by Verhoeven and Roenhorst (2000). Plants were assessed for disease symptoms
239 twice a week, and young non-inoculated leaves were tested by real-time RT-PCR 2-3 weeks
240 after inoculation. Twelve biological duplicates of samples with relatively low Cq-values were
241 used to inoculate three *Nicoticana benthamiana* and three *N. glutinosa* plants each.

242 Case study 3: Leaf material of *G. quadriradiata* and *S. oleraceus* was used to mechanically
243 inoculate *N. tabacum cv Xanthi*. Inoculum was made by homogenizing 1 g leaf material in 3
244 mL 0.1 M phosphate buffer (pH 7.8) using metallic beads. Test plant leaves were dusted with
245 carborundum (0.105 mm, VWR Chemicals) and 2 leaves of 3 plants per sample were
246 inoculated, after which leaves were rinsed with tap water. The experiment was performed in
247 a climate cell (PHCbi MLR-352H) under controlled conditions: 23°C (+/- 2°C), 10 h light period
248 and 19°C (+/- 2°C), 14h dark period and repeated twice.

249 Case study 6: Swab samples were inoculated onto a *Nicotiana tabacum* plant as described by
250 Skelton *et al.* (2023a).

251 Appropriate positive and negative inoculation controls were used in all case studies and
252 performed as expected.

253 Sequence analysis

254 Case study 4: The samples were processed using the Novel Enrichment Technique of
255 Viromes protocol (NetoVIR protocol (Conceicao-Neto *et al.*, 2015)), libraries were prepared
256 using the NexteraXT Library Preparation Kit (Illumina). Sequencing was performed using the
257 NextSeq500 platform (Illumina) for the leaf sample from location 1 and the NovaSeq6000
258 platform (Illumina) for the sample from location 2. The assigned sequencing clusters were
259 adjusted to achieve similar sequencing depth in both platforms, approximately ten million
260 reads per sample. The ViPER bioinformatics pipeline was used to process the sequencing
261 data (De Coninck, 2021). The reads were quality trimmed using Trimmomatic (Bolger *et al.*,
262 2014) and *de novo* assembly was performed using metaSPAdes (Langmead & Salzberg,
263 2012). The assembled contigs were classified using DIAMOND (Buchfink *et al.*, 2015) and
264 KronaTools (Ondov *et al.*, 2011) with the lowest common ancestor approach. The two
265 nucleotide sequences were aligned using MAFFT v7.453 (Katoh & Standley, 2013) and
266 visualized using an in-house R script. Phylogenetic analyses were performed using the

maximum likelihood method with TPM2u+F+G4 model using IQ-TREE (Nguyen *et al.*, 2015) with 1000 bootstrap replicates.

Results

Case study 1: ToBRFV detection in and around nonhost production greenhouses in the Netherlands

Case study 1 aimed to gain insight in the presence of ToBRFV at locations with and without prior ToBRFV outbreaks and in areas with high and low concentrations of tomato production. Samples were obtained within and around ToBRFV nonhost (cucumber or green bean) production greenhouses from areas with a high concentration of tomato production (Western area) and a low concentration of tomato production (Eastern area). Two greenhouse types were studied: those that switched from tomato to ToBRFV nonhost production in the past six months but were still under official ToBRFV control measures (Western area), and those that had not grown tomatoes for at least the past 4.5 years (Western and Eastern area). In the Western area, three companies (no. 2-4) also cultivated tomatoes in separate compartments with additional hygiene measures taken by the company to prevent cross-contamination between compartments. For two of those companies (no. 2 and 3), a few samples were also collected from the tomato compartments for comparison.

Western area

In the Western area with a high concentration of tomato production, ToBRFV was detected in multiple samples from all ToBRFV nonhost greenhouses as well as in one or more samples outside (Fig 2). Overall, ToBRFV positive samples from cucumber compartments of companies 1-4 with a ToBRFV outbreak in a recent previous tomato cultivation or with a current tomato cultivation in a nearby compartment showed lower Cq-values (Cq 13-39, mean Cq 24) than samples from companies 5-7 without recent tomato cultivation (Cq 23-31, mean Cq 29) (Fig 2a,c). However, Cq-values from company 1 (Cq 29-39, mean Cq 31) were relatively high, indicating a lower virus amount, and ToBRFV was detected in a lower number of samples than for companies 2-4 which were still producing tomatoes in different compartments. Cq-values from company 1 were more comparable to those of companies 5-7 which had not grown tomato in the past years. To assess whether there was a ToBRFV outbreak in the nearby tomato compartments of company 2 and 3, a few samples from young tomato leaves were tested and showed very low Cq-values (Cq 3), indicating an infection. Swab, soil, and water samples from the tomato compartments showed Cq-values comparable to those from cucumber compartments of the same companies (Fig 2b). In addition, a few samples were taken at three outside locations: around a hotel housing greenhouse staff, outside company 5, and across the road from a waste factory handling greenhouse waste. One or more positive samples were found at each location (Cq 21-39, mean Cq 29), including swabs from surfaces that are typically untouched (Fig 2d).

305 Furthermore, ToBRFV was detected from multiple swabbed surfaces that were exposed to
306 contact with individuals, plants, and objects in all sampled compartments, which might be
307 attributed to contact transmission. However, ToBRFV positive samples were also detected
308 from untouched surfaces at companies 2, 3, 4, 5 and 6. For instance, the ceiling frames
309 several meters above the mature plants tested positive for companies 2, 3, 4 and 6 (Fig 2a-
310 c). This indicated that ToBRFV residues also spread independently of contact, potentially
311 through aerosols. The outside of the glass ceiling panes yielded positive ToBRFV test results
312 for companies 2 and 3 (Fig 2a-b), whereas similar surfaces tested negative for the other
313 companies, potentially due to rainfall before sampling which might have washed away virus
314 residues. Interestingly, the inside surfaces of the glass ceiling panes consistently tested
315 negative, possibly due to a lack of dust accumulation on the lower side of the ceiling panes.

316 At the cucumber producing companies with a previous ToBRFV outbreak, ToBRFV was
317 detected on all leaf samples, both from leaves attached to living plants as from old leaves on
318 the floor (Fig 2a). All soil and rockwool samples tested positive as well. In contrast, at
319 companies without tomato cultivation in the past few years, only 4/12 cucumber leaf
320 samples and 2/3 soil and rockwool samples were ToBRFV positive (Fig 2c). For the water
321 samples, the only positive results were found for two wastewater samples from the tomato
322 compartments that had not yet undergone water disinfection treatment (Fig 2b).

323 Eastern area

324 In the Eastern area with a low concentration of tomato production, ToBRFV was detected
325 with relatively high Cq-values of at least Cq 28 in only a few samples from cucumber and
326 green bean production greenhouses (Fig 3a). At company 10, five hobby tomato plants were
327 grown in between the cucumber plants, of which leaf samples tested negative. Outside
328 company 10, leaves of three plant species, *Juglans regia, Pyrus communis* and *Poaceae*
329 species tested positive but with relatively high Cq-values of 24-32. All other samples taken
330 outside showed no amplification or Cq-values >32 (Fig. 3b). These results indicated that
331 there was less environmental ToBRFV contamination in the Eastern area than in the Western
332 area.

333 To assess infectiousness, biological duplicates of 4 leaf, 5 swab, and 3 soil samples with
334 relatively low Cq-values (tomato leaf: Cq 3, other samples: Cq 13-24) were inoculated onto
335 *N. benthamiana* and *N. glutinosa*. Samples originated both from cucumber and tomato
336 compartments in the Western Netherlands. Test plants only showed symptoms upon
337 inoculation by the tomato leaf sample and presence of ToBRFV was confirmed by real-time
338 RT-PCR (Table S1).

339 Case study 2: ToBRFV detection in tomato production greenhouses at different
340 outbreak stages in the Netherlands

341 To determine the infection status of three adjoining tomato production greenhouse units
342 belonging to the same company, leaf and sepal samples were tested for ToBRFV. Results
343 indicated that the three locations were in different phases of ToBRFV infection at the time of

344 sampling (Table 2). At location 1, a ToBRFV outbreak was present as the leaf and drain water
345 samples showed Cq-values of 6.5 and 22.9, and ToBRFV symptoms were observed in the crop.
346 In contrast, at location 2, sepal and water samples showed Cq-values around 33 and no
347 symptoms were observed. For location 3, sepal and water samples showed Cq-values of 23-
348 24, suggesting the potential start of an outbreak at the time of sampling, although no
349 symptoms were observed. To our knowledge, none of the sampled locations had been
350 previously infected with ToBRFV.

351 Swab samples were collected from different surfaces, with varying degrees of contact with
352 individuals, tomato plants, or objects, to determine the overall presence of ToBRFV at each
353 location (Table 3). The lowest Cq-values from swab samples were obtained from location 1
354 (Cq 12 – 36, mean Cq 21.8), followed by location 3 (Cq 19 – 36, mean Cq 28.3). At these
355 locations, ToBRFV was found on several surfaces that are typically untouched by individuals,
356 tomato plants, or objects, such as the lights (mean Cq 23.3), fan (mean Cq 21.4) (location 1),
357 and netting over the windows at the outside of the greenhouse (mean Cq 27.5) (location 3).
358 While ToBRFV was detected in swab samples from the smoking rooms (Cq 21-36, mean Cq
359 29), cigarette butts showed higher Cq-values (Cq 35-36, mean Cq 35), suggesting that
360 cigarettes were not the source of ToBRFV contamination in these rooms. The highest Cq-
361 values were obtained from location 2 (Cq 24 – undetermined, mean Cq 34.6). Although Cq-
362 values of tomato leaf samples from location 2 did not indicate an outbreak (Table 2), ToBRFV
363 was still detected on several surfaces, most notably on the concrete path (mean Cq 31.3),
364 groundsheet (mean Cq 30.9) and gutters (mean Cq 24.8). Overall, these results showed that
365 ToBRFV was detected at all three locations, even when plants had no symptoms and produced
366 high Cq-values.

367 Case study 3: ToBRFV detection on weeds outside tomato production greenhouses in 368 Belgium

369 To assess the presence of ToBRFV (residue) outside greenhouses, four weed species common
370 to Western Europe were sampled in the North of Belgium in an area with a high concentration
371 of tomato production. Samples were collected outside in the area immediately surrounding a
372 tomato production greenhouse with a likely ToBRFV outbreak. ToBRFV was detected in all
373 samples, with mean Cq-values ranging from 14.5 – 26.9 (Table 4). Cq-values (mean Cq 14.5 –
374 19.5) were lower for weeds that were sampled directly next to the greenhouse (touching the
375 outside of the greenhouse), in comparison to weeds that were sampled at a distance of
376 approximately 3 m (mean Cq 24.9 – 26.9). Mechanical inoculation leaf material from *G.*
377 *quadriradiata* and *S. oleraceus* onto the local lesion host *N. tabacum L. cv Xanthi* did not lead
378 to the appearance of lesions. Moreover, ToBRFV was not detected with the ToBRFV-specific
379 NFG-PCR in these two weed samples, indicating that the positive real-time RT-PCR results
380 were due to fragmented RNA.

381 Case study 4: Transmission of ToBRFV between adjoining greenhouses in Belgium

382 In case study 4, the sequences of two ToBRFV isolates infecting tomato in two neighbouring
383 greenhouse units of the same company were compared to investigate the possibility of

384 transmission through viral residues. At month 0, the first isolate was obtained at location 1
385 from plants that started showing ToBRFV symptoms and on which ToBRFV was detected (Cq
386 11) (Table 5, Fig S1a). Three months after symptoms were first observed, the crop was cleaned
387 out and the greenhouse was disinfected (Fig S1b). During crop clean-out, the infected plants
388 were put outside to dry, close to location 2, where due to weather conditions some of the
389 glass windows were open (Fig S1c). At month 3, the tomato plants at location 2 were not
390 showing any symptoms and were therefore not tested for ToBRFV. At month 5, two months
391 after th crop clean-out at location 1, the tomato crop at location 2 started showing symptoms
392 and ToBRFV was detected (Cq 6) (Table 5, Fig S1d).

393 Subsequent HTS analysis produced two nearly complete ToBRFV genomes of 6371 nt and 6377
394 nt (GenBank accession no. OQ633211 and OQ633212). Both sequences contained four open
395 reading frames encoding the viral small replicase subunit, RNA-dependent RNA polymerase
396 (RdRp), Movement Protein (MP), and Coat Protein (CP). Phylogenetic analysis of the protein
397 coding sequences indicated that these two isolates were 100% identical, but differed from
398 publicly available ToBRFV sequences, (Fig S2). These results provide additional evidence that
399 ToBRFV from location 1 served as the source of infection of the crop at location 2, presumably
400 through ToBRFV residues.

401 ## Case study 5: ToBRFV detection in a tomato production greenhouse in the UK

402 To investigate the detection of ToBRFV following a ToBRFV outbreak, swab samples were
403 taken from a range of different surfaces in a UK tomato production facility formed of two
404 conjoined greenhouses. ToBRFV was detected on all surfaces (Table 6), including some which
405 had not been in direct contact with individuals, plants, or objects, such as the top of
406 greenhouse lights, the inside of the fan units, and the entrance of the bee boxes. Of the 14
407 surfaces swabbed, 11 (79%) had mean Cq-values of less than 26, e.g. the roof structure (Cq
408 17.4), the ceiling lights in greenhouse 2 (Cq 25.3), the inside of the fans in both greenhouses
409 (Cq 17.5 and 22.1), and the entrances of bee boxes (Cq 22.9 and 17.0). All swab samples
410 taken from different areas of the greenhouse had some level of ToBRFV detection, indicating
411 the widespread presence of ToBRFV residue.

412 The Tyvek suit, worn by the person taking swabs, sampled from where the person had
413 leaned on the retaining bar on the scissor lift, had a mean Cq-value of 16.2. ToBRFV was also
414 detected on the gloves worn by the grower (mean Cq-value 19.8). These were clean from the
415 box when first put on, and no plant material was touched by the grower, but door handles
416 and other equipment had been touched. The leaf material from the greenhouse had a mean
417 Cq-value of 11.2 and could therefore have been a likely source of contamination.

418 ## Case study 6: ToBRFV detection before and after disinfection in an experimental
419 greenhouse in the UK

420 Swab samples were taken from different surfaces of a ToBRFV experimental quarantine
421 greenhouse to assess the effect of disinfection treatment. ToBRFV was detected on all
422 surfaces pre-hydrogen peroxide fogging treatment; mean Cq-values ranging from 19.8 (floor)

423 to 28.5 (bench). Post fogging, the Cq-values from most of the surfaces increased, especially
424 for the door and floor. Detection levels for the glass panes stayed mainly the same but the
425 Cq-values for the bench decreased slightly post fogging treatment (Table 7).

426 Swabs were also taken from the surfaces before and after disinfection and tested by
427 bioassay on *N. tabacum* to check for infectiousness. No virus symptoms were detected on
428 any of the inoculated plants.

429 Discussion

430 The case studies on ToBRFV presented here demonstrate the presence of detectable virus or
431 residues in diverse types of samples and settings from sites in the Netherlands, Belgium, and
432 the UK, both in the presence and absence of nearby infected plants. The detection of
433 ToBRFV from nonhost plants and inanimate surfaces clearly shows the extent to which
434 ToBRFV and/or its residues can spread beyond infected crops, highlighting the importance of
435 contextual information for interpreting positive test results.

436 Previous studies have shown that tobamoviruses were detected in both the broader
437 surroundings and in the immediate vicinity of crop production. Castello *et al.* (1995)
438 detected infectious tobacco mosaic virus in fog and clouds in New York State and Maine,
439 USA. In Slovenia, wastewater influent and effluent were shown to contain infectious pepper
440 mild mottle virus, tobacco mild green mosaic virus, and tomato mosaic virus (Bacnik *et al.*,
441 2020). Furthermore, in a commercial greenhouse with a high percentage of cucumber plants
442 infected by cucumber green mottle mosaic virus, contamination of many surfaces has been
443 shown after crop removal (Ellouze *et al.*, 2020). Additionally, a study at a ToBRFV-infected
444 tomato greenhouse in Germany showed that infectious ToBRFV was detected from swab
445 samples of different surfaces not only in the greenhouse, but also in the packaging hall and
446 staff accommodation, presumably as a result of cross contamination by human contact
447 (Ehlers *et al.*, 2023).

448 In addition to contact transmission, case studies 1, 2, and 5 indicated that ToBRFV residues
449 can also spread independently of contact, as swabs from surfaces that are typically not in
450 contact individuals, plants, or objects tested positive. Skelton et al. (2023b) hypothesized
451 that erratic detection of ToBRFV from non-inoculated control plants was due to
452 environmental contamination of the virus originating from highly infected plants in close
453 proximity. This environmental residue contamination may have included aerosols carrying
454 the virus or finely powdered dried material (dust) from infected plants. This hypothesis was
455 supported by the observation that at ToBRFV outbreak sites, lower Cq-values were observed
456 in samples from older leaves compared to younger leaves or sepals of asymptomatic plants,
457 which might be explained by the accumulation of dust (Skelton et al., 2023b).

458 Furthermore, the detection of ToBRFV in greenhouses producing a nonhost crop, which had
459 no prior history of tomato cultivation (case study 1), indicated that positive test results could
460 not be attributed to residual virus from a previous cultivation, but rather must have

461 originated from external sources. The often dusty outside of the glass ceiling panes tested
462 positive in some cases, whereas the non-dusty inside of the glass ceiling panes never tested
463 positive, not even from tomato compartments with an ongoing ToBRFV outbreak. This
464 supports the hypothesis that ToBRFV (residue) was spread through aerosols, containing the
465 virus or dried material from infected plants, such as leaf particles, pollen, and trichomes
466 (dust). The airborne dispersion of virus or viral residues could also explain the detection of
467 ToBRFV outside of greenhouses, as was found in case studies 1 and 3.

468 Proximity to a source of infection has been observed to positively affect the rate of ToBRFV
469 spread before (Ehlers et al., 2023). Indeed, the highest levels of ToBRFV were found in
470 samples from companies with a previous ToBRFV outbreak and adjacent tomato cultivation
471 in case study 1. Similarly, in case study 2 the highest levels of ToBRFV surface contamination
472 were found at the locations with the lowest Cq-values in tomato. In case study 3, weeds
473 sampled closest to a greenhouse with a likely ToBRFV outbreak also showed the lowest Cq-
474 values. For the companies without a previous ToBRFV outbreak or no adjacent tomato
475 cultivation, ToBRFV was detected from fewer samples and with higher Cq-values (case study
476 1). Presumably, these positive samples were due to the presence of ToBRFV residues in the
477 environment.

478 In case study 1, a high ToBRFV virus pressure was present at the time of sampling, since in
479 2023 ToBRFV was identified from many locations, predominantly in the Western part of the
480 Netherlands (https://nextstrain.nrcnvwa.nl/ToBRFV/20240205). In addition, genomic
481 analyses have indicated that at tomato production sites ToBRFV was presumably applied on
482 purpose to plants, even though it is a quarantine pathogen (Botermans *et al.*, 2023). The
483 combination of purposeful application of a quarantine virus and subsequent natural spread
484 may have resulted in a relatively high virus pressure in the Netherlands, especially in the
485 Western area.

486 Indeed, case study 1 showed the detection of ToBRFV across all sampled locations in the
487 Western Netherlands, both inside and outside greenhouses, indicating a widespread
488 presence of the virus or virus residues in the region. In the Eastern Netherlands, with a much
489 lower concentration of tomato production, ToBRFV was detected from fewer samples and
490 with clearly higher Cq-values than in the Western area, suggesting that a lower
491 concentration of inoculum in an area may lead to lower levels of environmental residues.
492 Overall, the limited number of samples in this study hindered statistical comparisons
493 between sample types and locations. However, there was a clear trend of more positive
494 samples, with a higher viral load for those locations that were in close proximity to tomato
495 production. Beyond the diagnostic risk of detecting ToBRFV in the absence of infected
496 plants, a high virus presence can also oppose the effectiveness of novel ToBRFV-resistant
497 cultivars, since this increases the likelihood of the emergence of ToBRFV isolates that can
498 break through resistances. The first mutant that could break through a newly introduced
499 ToBRFV-resistant cultivar has already been detected (Zisi *et al.*). Furthermore, the fact that

ToBRFV resistant cultivars currently on the market do not provide complete immunity further highlights the importance of achieving and maintaining a low virus pressure.

Due to the persistent nature of tobamoviruses, however, it is difficult to eradicate these viruses as they can remain infectious under extreme conditions and for extended periods of time (EPPO, 2024). ToBRFV has been found to remain infectious for at least 28 days on some surfaces such as aluminum and stainless steel and up to six months for plastics and glass (Skelton et al 2023a). Such variations in the survival rate on different surfaces have also been observed for potato spindle tuber viroid (Mackie et al 2015). These extended survival times potentially enable environmental residues to act as an inoculum source of future crops.

To gain further insight in the potential transmission of ToBRFV from environmental residues, bioassays were performed with biological duplicates of several nonhost leaf, swab, and soil samples from case study 1, exhibiting relatively low Cq-values. None of the inoculations, however, resulted in virus transmission, neither did the inoculations from weed samples in case study 3, nor the swab samples from different surfaces in case study 6. In contrast, case study 4 suggested possible transmission of ToBRFV between two neighbouring greenhouses from the same company in Belgium. Based on the timing of infection in the two greenhouses and the high degree of sequence similarity among the respective virus isolates, it is likely that transmission happened through aerosols from dried infected plant material. However, it cannot be fully ruled out that transmission occurred via other routes (EPPO, 2024), but workers and tools were separated for both greenhouses and rigorous hygiene measures were applied, so it is considered unlikely that contact transmission played a role in transmission of the virus. This case study shows that infectiousness of viral residues cannot be excluded. Even a very low rate of virus transmission from the environment to plants may lead to widespread infection due to the ease of transmission from one infected plant to another. This emphasizes the necessity of collecting contextual information regarding infection status, infection history, and sampling location to assist in result interpretation.

The potential of transmission through environmental residues highlights the importance of effective ways to inactivate ToBRFV and other contact transmissible pathogens from surfaces following an outbreak, and the need for effective overarching disinfection protocols being used as part of eradication measures (Skelton et al., 2023a). Case study 6 showed that even after disinfection with hydrogen peroxide based fogging treatment, ToBRFV was detected from surfaces such as doors and windows, even though the virus appeared no longer infectious. These findings further support the previous reports of detection of ToBRFV from a range of substrates and surfaces following disinfection (Skelton et al., 2023a, Davino et al., 2020).

The detection of ToBRFV on diverse plant samples collected outside greenhouses, raises questions on their role in the epidemiology of this virus. In both studies in the Netherlands (case study 1) and Belgium (case study 3), plants gave positive test results in real-time RT-PCR and negative results in bioassay. In addition, the results of the ToBRFV-specific NFG-PCR

539 tests in case study 3 indicated the absence of intact viral RNA. Given that the sampling in
540 case study 3 occurred near a company likely experiencing an ongoing ToBRFV outbreak, it is
541 plausible that the detection of ToBRFV on weeds could be attributed to the spread of virus
542 residues from this company and does not prove infection. Recent literature suggested that
543 the detection of ToBRFV from weeds indicates their natural host status (Cultrona *et al.*,
544 2024, Salem *et al.*, 2022a), although also in these studies it cannot be excluded that the
545 detection of ToBRFV on weeds resulted from contamination with ToBRFV residues present in
546 the environment. The host status of these species needs confirmation, and at present there
547 are no indications that weeds play a role as a ToBRFV reservoir.

548 Regarding diagnostics, the increased use of sensitive methods like real-time RT-PCR, enables
549 the detection of pathogens at relatively low concentrations. In the case of ToBRFV, however,
550 positive test results may either indicate actual (nascent) infections or the presence of
551 residual genetic material in the environment. The case studies here show the detection of
552 ToBRFV in leaf samples from nonhost plants and other sample types, probably due to
553 environmental ToBRFV residues, which raises the possibility that healthy, uninfected tomato
554 plants also yield positive test results. This presents diagnostic challenges when dealing with a
555 stable virus like ToBRFV, especially in areas with lots of environmental residues. Therefore,
556 depending on the purpose of testing, Cq-values may need to be interpreted in conjunction
557 with contextual information, such as the origin of the sample, symptoms observed, the
558 probability of residues from prior cultivations and the likelihood of residues in the broader
559 environment. This study highlights the complexity of decision-making in response to positive
560 test results and underscores the need for a nuanced approach to manage the impact of
561 ToBRFV in agricultural settings, together with further research to relate ToBRFV detection
562 data to the risks of creating new infections.

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574 Data availability statement

575 The nucleotide sequences from case study 4 can be found in GenBank
576 (https://www.ncbi.nlm.nih.gov/genbank/) with GenBank accession no. OQ633211 and
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Tables

Table 1. Number and types of samples per location and area.

		Western area: high concentration of tomato production												Eastern area: low concentration of tomato production								
		Cucumber greenhouse recently switched from tomato to cucumber cultivation, under ToBRFV measures.						Cucumber greenhouse with no tomato cultivation >4.5 years			Outside			Cucumber greenhouse with no tomato cultivation >4.5 years				Outside				
		Company						Company						Company								
		1	2[a]		3[a]		4[a]	5	6	7	company 5	across the road waste factory	hotel greenhouse staff	8	9	10	11	company 8	parking area	company 9	company 10	company 11
Sample type	Sample description	c	c	t	c	t	c	c	c	c				c	c	c	b					
Swab	Surfaces usually untouched by individuals, plants or	3	3	2	3	3	3	4	3	3	2	2	2	3	3	3	4	2	3	3	2	3
	Surfaces sometimes/often in contact with individuals,	4	3	1	3	1	5	4	3	5	-	1	1	5	5	5	4	2	1	1	2	1
Leaf	Mixed sample from the same species - living plants	2	1	1	1	1	1	2	2	2	2	1	-	2	2	3	4	3	3	3	3	3
	Mixed sample from the same species - dead leaves on	-	1	-	1	-	1	2	2	2	-	-	-	2	2	2	2	-	-	-	-	-
	Mixed sample from 2 volunteer tomato plants in	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Other	Soil below plastic covers greenhouse floor	-	1	1	1	-	1	-	1	-	-	-	-	1	1	1		-	-	-	-	-
	Substrate with plant roots	-	-	-	-	-	1	1	-	1	-	-	-	1	1	1	1	-	-	-	-	-
	Paperwork from ToBRFV outbreak or current tomato	1	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	Clean water	-	-	-	-	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-
	Rainwater	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
	Wastewater	1	1	1	1	1	1	1	1	1	-	-	-	1	1	1	1	-	-	-	-	-
Total number of samples		11	10	7	10	7	15	15	14	15	4	4	3	15	15	16	17	7	7	7	7	7

[a]Company 2, 3 and 4 also produced tomatoes in a different compartment. Tomato compartments were sampled for company 2 and 3. b: (green) bean compartment, c: cucumber compartment, t: tomato compartment

678 **Table 2**. Detection of ToBRFV in tomato leaf and sepal and water samples of different greenhouse
679 entities.

Location	Sample type	Cq-value[a]	Symptoms observed
1	Leaf	6.5	Yes[b]
	Water	22.9	
2	Sepal	33.4	No
	Water	33.3	
3	Sepal	22.9	No
	Water	23.9	

680

681 [a] Mean Cq-value obtained with CaTa28 and CSP1352 primers and probes.
682 [b] Symptoms involved mosaic and deformation in the head of the plant, and reduced leaf biomass;
683 only observed in a couple of rows at the time of sampling.
684

685 **Table 3.** Results of swab samples taken from different surfaces with varying degrees of
686 contact with individuals, plants, or objects.

Surface	ToBRFV real-time RT-PCR		
	Location 1	Location 2	Location 3
	Cq-value[b]		
Inside the greenhouse			
Concrete path	15.8	31.3	22.2
Groundsheet	18.2	30.9	24.1
Electric car	12.8	33.9	21.3
Electricity cabinet	20.2	35.6	19.5
Gutter	18.5	24.8	33.4
Pipe rail	15.7	35	21.3
Dripper	18.2	38.4	33.4
Bikes	23.2	N.s.	23.4
Lights[a]	23.3	36.1	34.4
Fan[a]	21.4	N.s.	N.s.
Outside the greenhouse			
Netting on outside of greenhouse[a]	N.s.	N.s.	27.5
Crates after harvest	25.4	N.s.	N.s.
Disinfected crates (before entry into greenhouse)	N.s.	N.s.	28.4
Canteen	26.3	37.1	30.5
Mobile phone, keys, badge, glasses	30.1	Undet.[d]	36.1
Office	24.1	35.9	32.6
Smoking room	20.8	35.7	30.1
Cigarette butt[c]	36.2	N.s.	34.5

N.s. = Not sampled.

[a] Surfaces untouched by individuals, plants and objects.

[b] Mean Cq-value obtained with CaTa28 and CSP1352 primers and probes

[c] Cigarette butt itself tested

687 [d] No signal obtained with real-time RT-PCR
688

689

690

691 **Table 4.** Cq-values of weed leaf samples taken outside a tomato production greenhouse in an area
692 with a high concentration of tomato production in Belgium.

Sample	Cq-value[a]	ToBRFV NFG-PCR
Sonchus oleraceus	14.5	-
Galinsoga quadriradiata	19.5	-
Solanum nigrum [b]	26.9	n.t.
Erigeron canadensis [b]	24.9	n.t.

-: No detection, n.t.: not tested

[a] Mean Cq-value obtained with CaTa28 and CSP1352 primers and probes

693 [b] Weeds sampled at approximately 3 m distance from the greenhouse (not touching the outside of the greenhouse)

694 **Table 5.** Detection of ToBRFV in tomato leaf samples of two adjoining tomato production greenhouse
695 units in Belgium.

Location	Month	Cq-value[a]	Symptoms observed
1	0	11.1	Yes[b]
2	5	5.8	Yes[b]

[a] Mean Cq-value obtained with CaTa28 and CSP1352 primers and probes.

696 [b] Symptoms observed involved yellow mosaic, deformation, and needle-tips on the leaves. No symptoms were observed on the fruits.
697

698 **Table 6.** ToBRFV real-time RT-PCR results of swab samples taken from different surfaces with varying
699 degrees of contact with individuals, plants, or objects at the UK tomato production greenhouse.

Surface	Mean Cq-value
Greenhouse 1	
Top of light[a]	35.4
Fan[a]	22.1
Underside gutter	20.9
Behind whiteboard	33.6
Top of cabletray[a]	20.3
Bumble bee box entrance[a]	22.9
Inside panel door	23.4
Socket lid	25.6
Pheromone trap	31.6
Greenhouse 2	
Roof structure[a]	17.4
Bumble bee box entrance[a]	17
Top of light[a]	25.3
Fan[a]	17.5
Underside gutter	16.8
General	
Tyvek Suit	16.2
Grower's Phone	31.2
Grower's Glove	19.8
Leaf material	11.2

700 [a] Surfaces untouched by individuals, plants and objects.

701

702

703

Table 7. ToBRFV real-time RT-PCR results of swab samples taken from different surfaces of the UK experimental greenhouse, pre and post a hydrogen peroxide based fogging treatment.

Surface	Mean Cq-value			
	Pre fogging treatment	Post fogging treatment 1	Post fogging treatment 2	Post fogging treatment 3
Aluminium door	26.8	31.5	31	30.6
Glass pane	23.7	24.4	24.8	21.5
Concrete floor	19.8	40	22.5	40
Steel bench	28.5	25.1	22.7	26.4
Aluminium ladder	23.1	22.2	23.7	26.2
Aluminium lance	25.7	25	31.8	24.4

Figures

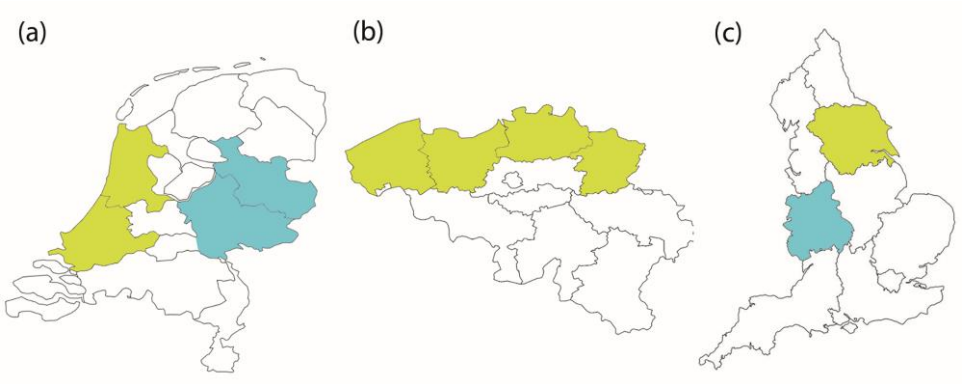
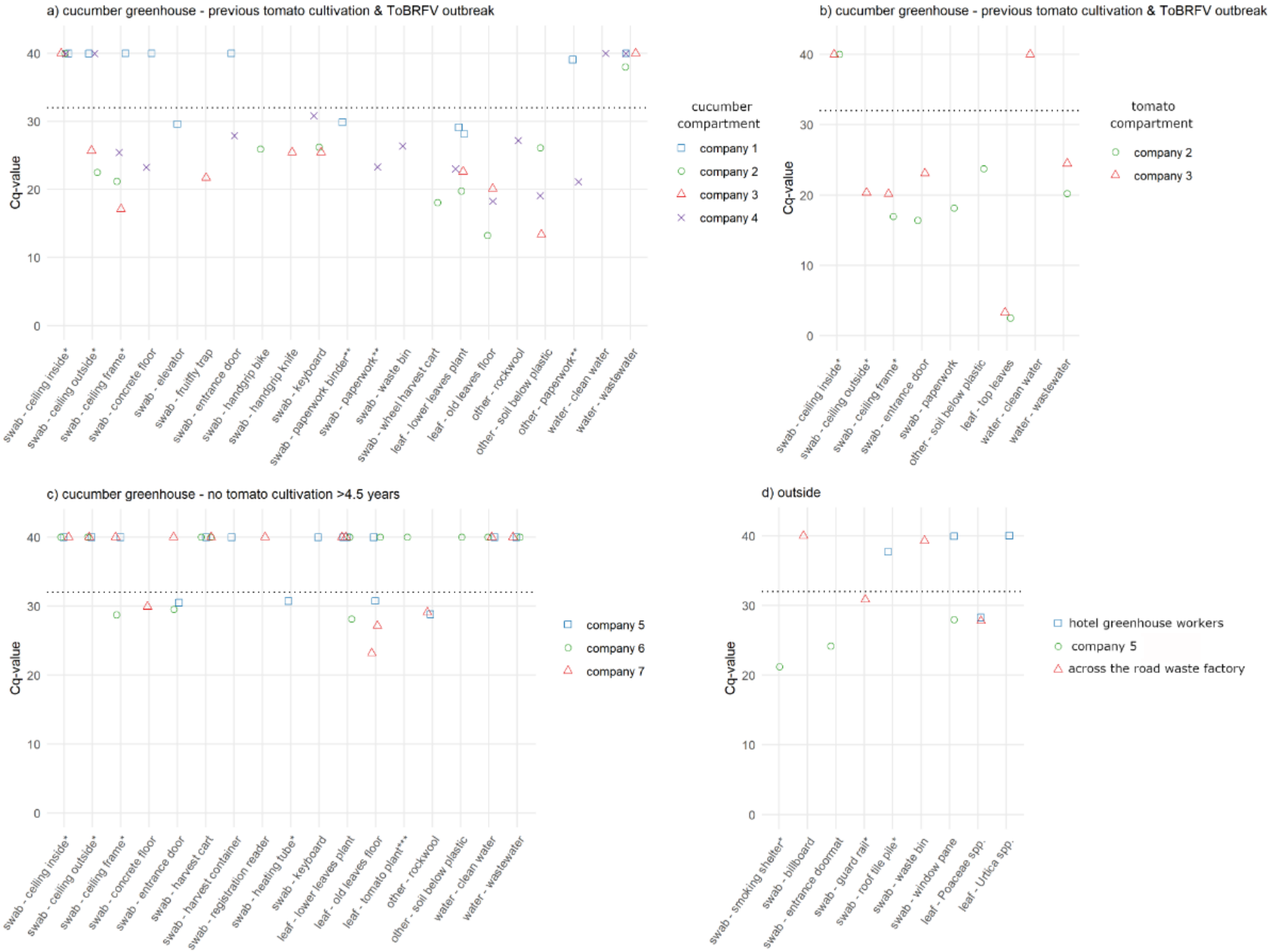


Figure 1. Regions of sampling a) West of the Netherlands (yellow; case study 1 and 2), East of the Netherlands (blue; case study 1) b) North of Belgium (yellow; case study 3 and 4) c) West Midlands, UK (blue; case study 5), North-East of England, UK (yellow; case study 6). Maps are not drawn to scale. Companies/locations were unique for each case study, exact geographic locations were omitted for confidentiality reasons.


a) cucumber greenhouse - previous tomato cultivation & ToBRFV outbreak
Cq-value
40
30
20
10
0
swab - ceiling inside*
swab - ceiling outside*
swab - ceiling frame*
swab - concrete floor
swab - elevator
swab - fruitfly trap
swab - entrance door
swab - handgrip bike
swab - handgrip knife
swab - keyboard
swab - paperwork binder**
swab - paperwork**
swab - waste bin
swab - wheel harvest cart
leaf - lower leaves plant
leaf - old leaves floor
other - rockwool
other - soil below plastic
other - paperwork**
water - clean water
water - wastewater
cucumber compartment
company 1
company 2
company 3
company 4
b) cucumber greenhouse - previous tomato cultivation & ToBRFV outbreak
Cq-value
swab - ceiling inside*
swab - ceiling outside*
swab - ceiling frame*
swab - entrance door
swab - paperwork
other - soil below plastic
leaf - top leaves
water - clean water
water - wastewater
tomato compartment
company 2
company 3
c) cucumber greenhouse - no tomato cultivation >4.5 years
Cq-value
swab - ceiling inside*
swab - ceiling outside*
swab - ceiling frame*
swab - concrete floor
swab - entrance door
swab - harvest cart
swab - harvest container
swab - registration reader
swab - heating tube*
swab - keyboard
leaf - lower leaves plant
leaf - old leaves floor
leaf - tomato plant***
other - rockwool
other - soil below plastic
water - clean water
water - wastewater
company 5
company 6
company 7
d) outside
Cq-value
swab - smoking shelter*
swab - billboard
swab - entrance doormat
swab - guard rail*
swab - roof tile pile*
swab - waste bin
swab - window pane
leaf - Poaceae spp.
leaf - Urtica spp.
hotel greenhouse workers
company 5
across the road waste factory

715

716 **Figure 2.** Cq-values of samples from the area with a high concentration of tomato production (Western Netherlands). a) Cucumber
717 compartment of greenhouses with a previous ToBRFV outbreak, that switched to cucumber cultivation in all or some compartments.
718 Companies 2-4 were still producing tomatoes in a different compartment. b) Tomato compartments of company 2 and 3, samples were taken
719 for comparison with the cucumber (nonhost) compartments c) Greenhouses with no previous tomato cultivation in at least the past 4.5 years.
720 d) Outside locations. Dotted line: threshold below which samples are considered positive for official samples in the Netherlands. *Surfaces
721 typically untouched by indiviuals, plants and objects. ** Paperwork from the ToBRFV outbreak when tomato was grown, sampled in a room
722 next to the cucumber compartment. ***A young volunteer tomato plant at the edge of the plastic covering the soil at company 6. Cq 40: no
723 amplification. Cq-values refer to the CaTa28 primers and probes, comparable results were obtained for CSP1325 and controls reacted as
724 expected. Points were plotted with a jitter function to prevent overlap.

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726

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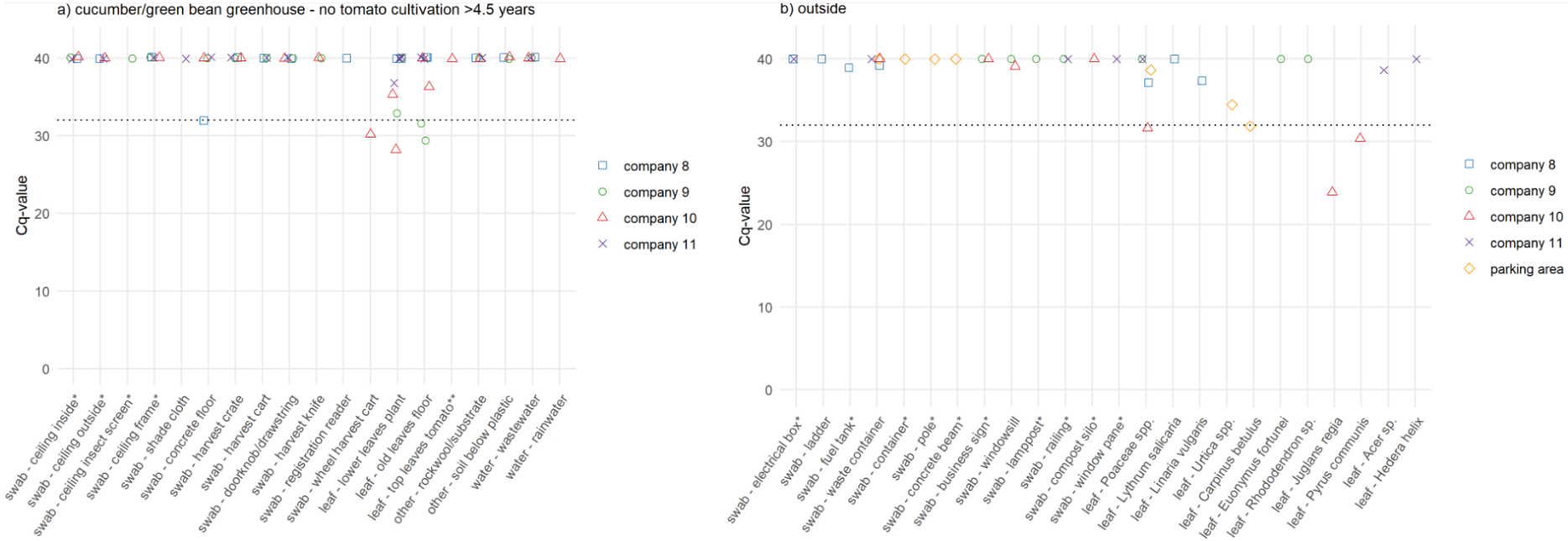

Figure 3. Cq-values of samples from the area with a low concentration of tomato production (Eastern Netherlands). a) Cucumber or green bean compartment of greenhouses with no previous tomato cultivation in at least the past 4.5 years. b) Outside locations. Dotted line: threshold below which samples are considered positive for official samples in the Netherlands. *Surfaces typically untouched by individuals, plants and objects. ** five hobby tomato plants grown in between the cucumber plants. Cq 40: no amplification. Cq-values refer to the CaTa28 primers and probes, comparable results were obtained for CSP1325 and controls reacted as expected. Points were plotted with a jitter function to prevent overlap.

Supporting information

Table S1. Symptoms and Cq-values on *Nicotiana benthamiana* and *N. glutinosa*, after inoculation with samples of which biological duplicates showed relatively low Cq-values (case study 1).

				Cq value of biological duplicate sample		ToBRFV symptoms		Cq-values (non-inoculated young leaves, 2-3 weeks after inoculation)			
								N. benthamiana (n=3)		*N. glutinosa* (n=3)	
Company	Compartment	Sample nr	Sample type	CaTa28	CSP1325	N. benthamiana (n=3)	N. glutinosa (n=3)	CaTa28	CSP1325	CaTa28	CSP1325
2	cucumber	4	Leaf - old leaves floor	13.23	14.205	-	-	-	-	-	-
2	tomato	12	Swab - ceiling frame	16.965	18.11	-	-	-	-	-	-
2	tomato	13	Leaf - top leaves	2.515	3.27	systemic: rugosity, leaf curling, chlorosis, growth inhibition	local: necrotic lesions	3.08	2.55	32.24	33.175
2	tomato	15	Other - soil below plastic	23.74	24.12	-	-	-	-	-	-
2	tomato	17	Swab - entrance door	16.425	18.06	-	-	-	-	-	-
3	cucumber	2	Swab - ceiling frame	17.125	19.68	-	-	-	-	-	-
3	cucumber	4	Leaf - old leaves floor	20.095	21.09	-	-	-	-	-	38.58
3	cucumber	8	Other - soil below plastic	13.38	14.975	-	-	-	-	33.07	38.82
3	tomato	12	Swab - ceiling frame	20.185	21.83	-	-	-	37.41	-	-
3	tomato	13	Swab - ceiling outside	20.34	22.15	-	-	-	-	-	-
4	cucumber	9	Leaf - old leaves floor	18.26	19.145	-	-	-	-	-	-
4	cucumber	10	Other - soil below plastic	19.09	18.415	-	-	-	-	-	-

-: no symptoms or no detection

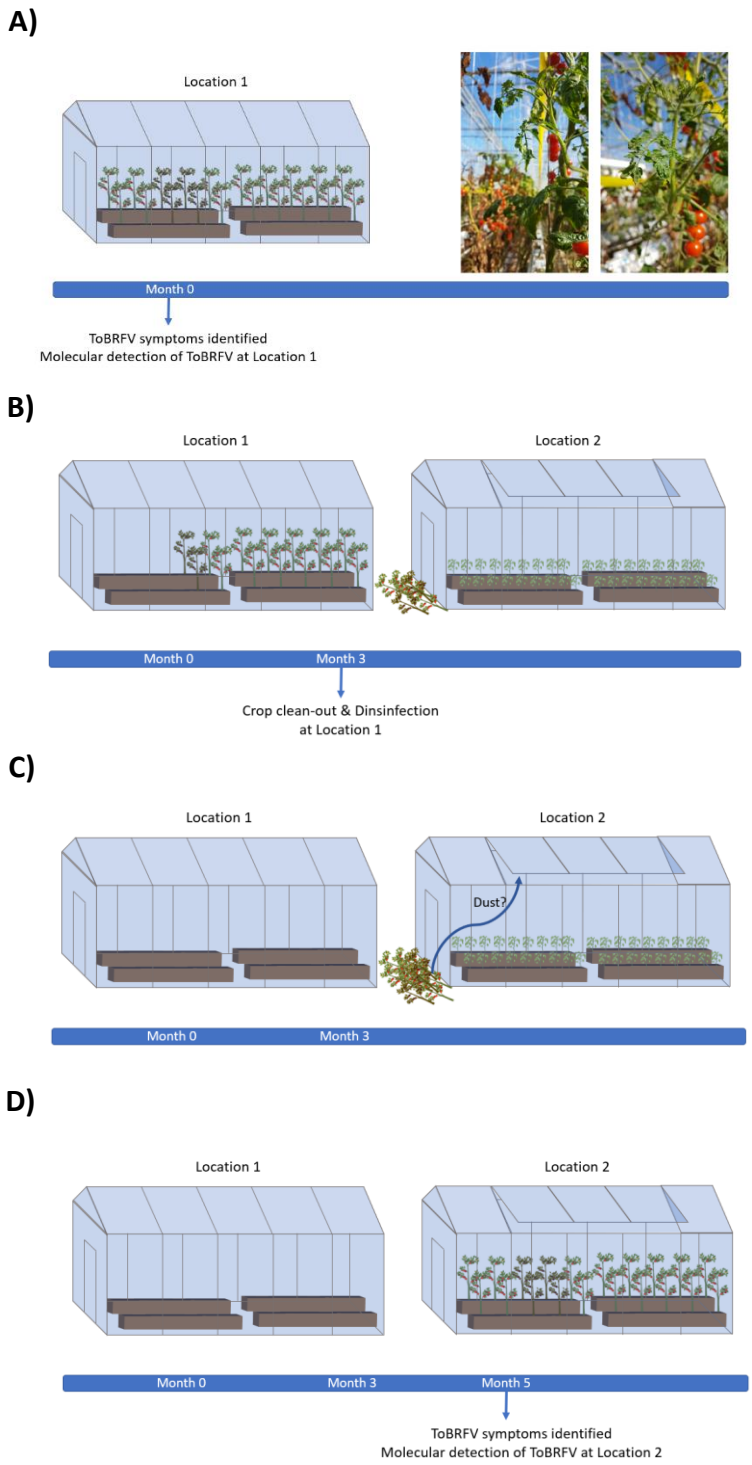

Figure S1: Hypothesis for transmission of ToBRFV through dust (case study 4). a) At location 1, ToBRFV symptoms were identified (Month 0). Infection was confirmed by real-time RT-PCR. b-c) Three months later, the ToBRFV-infected crop at location 1 was cleaned out and the greenhouse was disinfected. The cleaned-out crop was put outside to dry, close to location 2. Location 2 had opened some of its windows due to weather conditions, allowing dust from the decomposing infected crop of location 1 to enter the greenhouse. d) At month 5, ToBRFV symptoms were identified at location 2. Infection was confirmed by real-time RT-PCR. Figures were created with Biorender.com.

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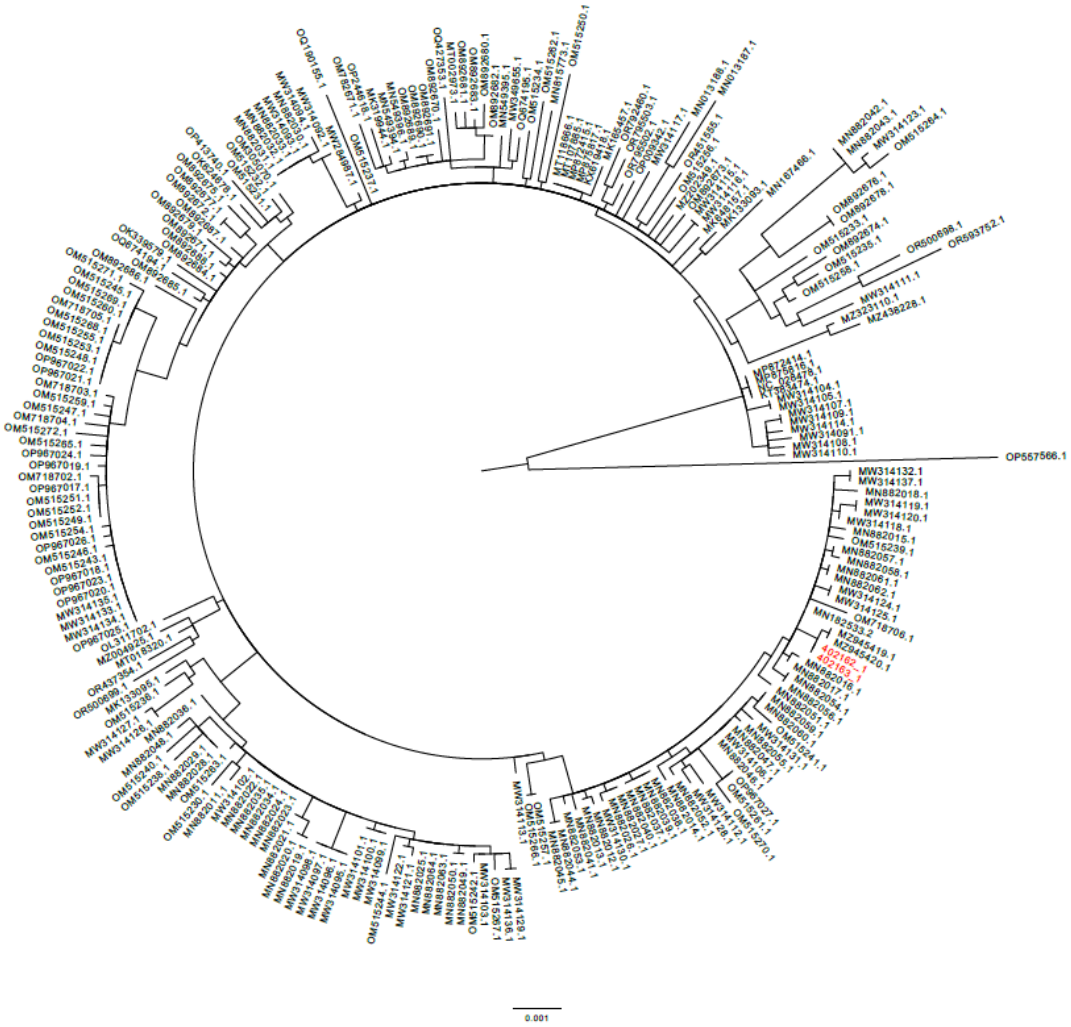

Figure S2. ToBRFV phylogenetic analysis based on the protein coding sequences (case study 4). Maximum-likelihood tree of the ToBRFV isolate sequences generated in this study are indicated in red (Isolate 402162_1 (GenBank accession no. OQ633211) and 402163_1, (GenBank accession no. OQ633212), whereas publicly available complete genome sequences from NCBI (accessed on 04/01/2024) are indicated in black.