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ORIGINAL ARTICLE

Detection of tomato brown rugose fruit virus is influenced by infection at different growth stages and sampling from different plant parts

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Abstract

Since the first report of the virus in 2014, tomato brown rugose fruit virus (ToBRFV) has spread widely through Europe, the Americas and Asia. Within Europe there is currently a requirement for annual surveillance for the virus. However, little is known about the relative impact of sampling strategy with respect to timing of infection and the detection of virus from different plant parts. To test reliably for ToBRFV in crops of unknown infection status, this issue needed to be addressed. To do this, two different approaches were followed: (1) inoculation experiments were conducted at two institutes to look at the relative effects of time of infection, plant parts, cropping season and cultivar on detection of the virus; and (2) sampling and testing various plant parts were carried out during active outbreaks from two tomato production sites in the Netherlands to look at the effect of sampling plant parts on detection of the virus. In inoculation experiments, the greatest impact on detection was timing of infection, with plants infected early in the growth cycle showing a predictable development of infection. In plants infected later, infection was detectable in sepals (calyx) earlier than in older leaves. In the studies carried out on commercial crops during ToBRFV outbreaks, the highest virus concentrations were obtained from testing sepals and young leaves. Thus, in a young crop where sepals and fruit are not yet developed, sampling should focus on the young leaves; in a mature crop it may be better to sample sepals and/or fruit.

KEYWORDS
detection, fruit, plant parts, sepals, tomato brown rugose fruit virus

1 | INTRODUCTION

Tomato brown rugose fruit virus is a member of the genus *Tobamovirus*. Following the initial detection of tomato brown rugose fruit virus (ToBRFV) in Jordan in 2014/2015 (Salem et al., 2016), the virus was subsequently also reported from Israel (Luria et al., 2017). The virus has since rapidly emerged as a risk to commercial tomato and pepper production around the globe because it is able to overcome the *Tm-2*^{2} resistance gene that provides resistance to several tobamoviruses in tomato (*Solanum lycopersicum*) (European and Mediterranean Plant Protection Organization [EPPO], 2022a). Due to the risk to tomato and pepper (*Capsicum* spp.) crops, the virus is the focus of quarantine

control measures and is under eradication in many countries where it has been detected. Infected crops may suffer from reduced yield and fruit appearance may be affected, resulting in loss of marketable yield (EPPO, 2022a).

ToBRFV, like other tobamoviruses, is mechanically transmitted. It has also been demonstrated to be seedborne and may be spread by bumblebees (Davino et al., 2020; Levitzky et al., 2019; Salem et al., 2022). Once the virus has infected a plant in a susceptible crop, it can spread through normal working practices. It has been shown to infect a whole crop within a single cropping cycle under experimental conditions (Panno et al., 2020). As with other environmentally stable, contact–transmitted pathogens where resistant varieties are not available, control of the virus relies on prophylactic biosecurity measures, such as testing of seed and application of hygiene best–practice measures (European Food and Safety Authority, 2011), or eradication following an outbreak. The virus is stable, can remain infectious for at least 6 months in dried sap, and is resistant to many disinfectants used on both seed and a range of glasshouse surfaces (Chanda, Shamimuzzaman, et al., 2021; Davino et al., 2020; Samarah et al., 2021; Skelton et al., 2021). To support surveillance inspections of plants and seeds, it is important to be able to detect the virus reliably. Multiple diagnostic methods have been developed, including serological tests using ELISA and molecular tests such as (real-time) reverse transcription–PCR, loop-mediated isothermal amplification (LAMP) and cluster regularly interspaced short palindromic repeats (CRISPR) (Alkowni et al., 2019; Alon et al., 2021; Bernabé-Orts et al., 2021; Chanda, Gilliard, et al., 2021; Fidan et al., 2021; International Seed Federation, 2020; Levitzky et al., 2019; Menzel & Winter, 2021; Yan et al., 2019). Some of the molecular tests have been validated for regulatory use and are recommended in international standards and for regulatory diagnostic activities such as the European Union Emergency Measures (Commission Implementing Regulation [EU] 2020/1191, amended by Commission Implementing Regulation [EU] 2021/74 and Commission Implementing Regulation [EU] 2021/1809) (EPPO, 2022a).

Two key aspects should be considered when sampling from a potentially infected crop: the likely distribution of the pest/pathogen and the diagnostic efficacy of the test being used. While a great deal of research has been focused on developing and validating detection tests, less research focus has been given to the relative influence of sampling on diagnostic outcome, that is, how many plants and which host tissues (plant parts) to sample to maximize the potential for detection. Recommended sample sizes for regulatory inspections are laid out within International Standards on Phytosanitary Management (ISPM), standard 31 'Methodologies for Sampling of Consignments' (International Plant Protection Convention, 2008), with consideration being given to the thresholds of detection afforded by a given number of samples from a consignment. However, the concentration of the target pathogen in various plant parts, and consequently the choice of sampled tissue, will also have major influence on the outcome of the test. Currently, little is known about the in-plant distribution of ToBRFV with respect to time after infection and plant age. Samuel (1934) tracked the movement of the closely related tobacco mosaic virus (TMV) through tomato plants using an approach of sectioning infected plants and testing them using a bioassay. This was repeated in young and mature plants. In young plants, it was found that the virus could first be detected in the roots, before moving to the top of the plant, and eventually infecting every leaf. In contrast, in mature plants, the virus could first be detected in the roots and then the top of plants, but the plant was never fully systemically infected and detection was erratic (Samuel, 1934). It is recognized that a full systemic infection of plants by a virus does not always occur, and this asymmetric infection is noted more in viruses that move inefficiently. Furthermore, even when fully systemic infection is achieved, the virus accumulates to different levels within the plant, the highest virus concentration being found in symptomatic and developing leaves/stem (Hull, 2014). Because viruses may be unevenly distributed through plants, the choice of where to sample for a diagnostic test is crucial. Moreover, insight into which plant parts are most suitable for detection leads to well-informed pragmatic choices when designing surveillance strategies.

The aim of the studies reported here was to support the formulation of practical sampling information for plant health authorities during surveillance for ToBRFV.

2 | MATERIALS AND METHODS

Three independent studies are reported here. Inoculation experiments 1 and 2 (IE1 and IE2) were controlled experiments conducted under biosecure quarantine conditions at Fera Science Ltd, York, UK, and Wageningen University & Research (WUR), Netherlands, respectively. These aimed to investigate the detection of ToBRFV from different plant parts over time with respect to time of inoculation, cultivar and, in IE1, also in cropping cycle and growing conditions. In the third study, hereafter referred to as 'outbreak comparison' (OC), samples were collected from different plant parts of infected crops during active ToBRFV outbreaks in two tomato production sites in the Netherlands. In all studies, plants were tested using reverse transcription–quantitative real–time PCR (RT–qPCR) to allow comparison of relative viral load in different plant parts to support improved sampling strategies for the detection of ToBRFV. Additionally, ELISA was used in IE2 for comparison with RT-qPCR.

2.1 | Inoculation experiment 1 (IE1, Fera, UK)

2.1.1 | Virus isolates and inoculation

All glasshouse trials were set up with the same basic format across four treatments:

- Winter 'lit' crop (initiated 04 November 2020).
  - Glasshouse 1: early inoculation on entry to glasshouse (04 November 2020).

- Glasshouse 2: late inoculation after 9 weeks in glasshouse (06 January 2021).
- Spring 'unlit' crop (initiated 21 April 2021).
  - Glasshouse 3: early inoculation on entry to glasshouse (21 April 2021).
  - Glasshouse 4: late inoculation after 9 weeks in glasshouse (16 June 2021).

To avoid cross contamination between trials, each treatment was sited in a different glasshouse, but under identical conditions. For each treatment, plants were approximately 8 weeks old when brought into the glasshouse. Four plants of each of two commonly grown commercial varieties (cv.) representing different fruiting types, cv. Piccolo (cherry) and Roterno (intermediate-round), grafted onto cv. Maxifort, were grown in a mock-hydroponic set-up, with each plant being of alternating variety. At the point of inoculation, early inoculated plants were approximately BBCH growth stage 29, and late inoculated plants approximately BBCH growth stage 76 (Meier, 1997). Additionally, two healthy control plants, one of each variety, were included in a separated mock-hydroponic set-up and were processed and tested by the same methods as the inoculated plants. Plants were grown in insect-proof glasshouse cubicles under an appropriate plant health quarantine licence. The set–up was sterilized to mitigate against inadvertent contamination with ToBRFV following procedures from Skelton et al. (2021). Bumblebees were not used for pollination to avoid contamination between plants; a small paintbrush was used instead, with a different brush for each infected repetition and for healthy plants. In all treatments the photoperiod was 16 h light/8 h dark with temperature maintained at 22°C (day) and 18°C (night).

Prior to inoculation, top leaves of all plants were tested for ToBRFV using RT-qPCR to ensure they were virus-free (see section on IE1 ToBRFV screening below). Plants were inoculated with a commercially available ToBRFV isolate (DSMZ, PV-1236), which had been multiplied in tomato plants of cv. Moneymaker, at a 1:1000 dilution. One compound leaf of each plant, approximately one-third of the way up the plant (approximately 0.5 m from the base of the plant), was mechanically inoculated with the diluted isolate and celite following standard procedures (EPPO, 2022b). Due to the persistence of the virus, to avoid inadvertent sampling from the inoculated leaf, inoculated leaves were marked by tying a piece of nylon twine around the petiole of the whole compound leaf, ensuring these leaves were not sampled later in the experiment.

2.1.2 | Sample collection

Leaf

Leaf samples were taken from all plants across set time points: at Days 2, 5, 7, 9 and 12, then weekly from Weeks 2 to 12 and finally, every 2 weeks at Weeks 14, 16, 18 and 20. Sample collection was discontinued when plants were no longer fit for testing, generally when leaves became dried and necrotic. At each time point, leaf samples were taken from the top, middle and bottom of each plant (Figure 1) with gloves being changed between each sample. It is important to note that the same leaf was not sampled each time but leaves from the same region on the plant.

Sepals (calyx)

Samples of sepals were taken from plants as they developed and the specific time point and plant location was recorded. These were only collected from ripe fruits.

Fruit

Tomato fruit were taken from plants in all four glasshouses as they were ripe. In winter crop treatments, these were taken at two time points, but in spring crop treatments, these were picked throughout the growth period with time point and sample location being recorded.

In each case, samples were stored at −80°C until tested.

2.1.3 | ToBRFV screening

Representative subsamples of plant material (leaf, sepal or fruit), with guanidine hydrochloride (GH+) buffer (EPPO, 2022a) 1:10 weight to volume, were ground using a HOMEX 6 (Bioreba) and RNA was extracted by magnetic bead extraction using Invimag Virus DNA/RNA mini-kit (Invitek GmbH). The RNA extracts were stored at −20°C.

RT-qPCR was performed using iTaq universal probes one-step reaction mix (Bio-Rad) containing 1 µL of total RNA extract. All samples were initially tested for cytochrome oxidase (*COX*) (Weller et al., 2000) as an internal control, then run in duplicate wells for ToBRFV (EPPO, 2022a; Menzel & Winter, 2021). All testing for ToBRFV was carried out on a QuantStudio 6 Flex real-time PCR system according to the manufacturer's instructions.

The average cycle threshold (C_t) value between the two wells was recorded for each sample and it was compared chronologically for leaves, and where applicable sepals, fruits, side shoots and roots. Where interpretation of a 'positive/negative' test outcome was required, if test reactions gave a C_t value of ≤32, the virus was regarded as present. These thresholds were set in line with thresholds applied in the OC trial (see Section 2.3.2).

2.2 | Inoculation experiment 2 (IE2, WUR, Netherlands)

2.2.1 | Virus isolate and inoculation

Tomato plants of two commercial cultivars (Merlice and Brioso) grafted onto Maxifort, were grown in a small glasshouse compartment with 16 h light/8 h dark at a temperature of 20°C and 80% relative humidity. Pollination was not carried out in this experiment.

For each cultivar, six plants were inoculated 4 weeks after sowing, at approximately BBCH growth stage 17 (Meier, 1997), with a Dutch isolate of ToBRFV (NVWA 33610411, NCBI accession code MN882011) propagated on tomato cv. Moneymaker. Inoculum was

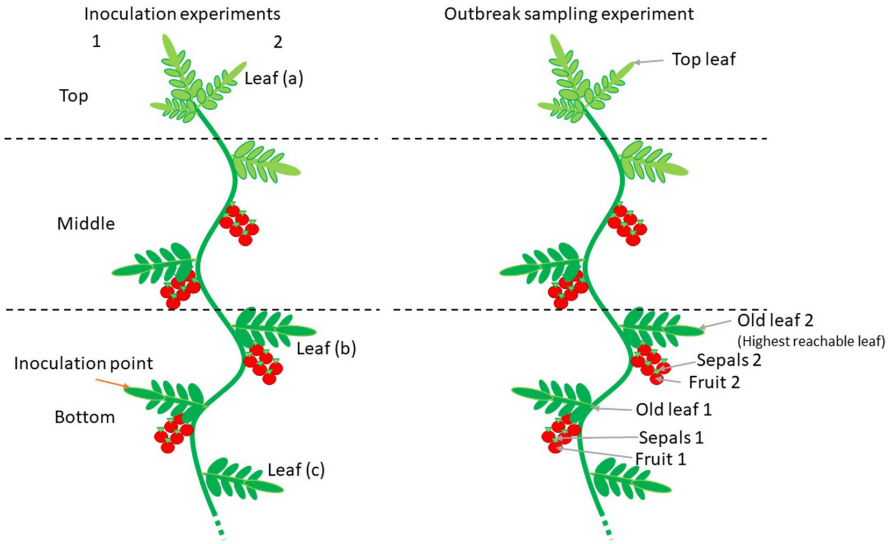

FIGURE 1 Diagram showing sampling points from tomato plants in inoculation experiments and the outbreak sampling comparison for detection of tomato brown rugose fruit virus. For the inoculation experiments (left), leaf samples were taken from the top, middle and bottom segment of each plant. In inoculation experiment 2 (IE2), these were labelled a, b and c. In IE2, after 21 days, sepals rather than leaves were sampling point c. Samples taken for the outbreak sampling comparison (OC) are indicated on the right. At Site 2 of the OC the plant parts were sampled from lowest to highest expected viral load as follows: lowest old leaf, old leaf approximately 2 m from the ground, lowest sepals from lowest truss, lowest fruit from lowest truss, lowest sepals from second lowest truss, lowest fruit from second lowest truss and young leaf from top of the plant; the sepals belonged to the sampled fruits. At Site 1, no fruits were sampled, but samples were taken in the same order (from lowest to highest expected viral load).

prepared by grinding infected tomato leaves showing clear ToBRFV symptoms in 0.03 M Na-K-phosphate buffer pH 7.2. The optimal inoculum dilution of 1:50 dilution in 0.03 M Na-K-phosphate buffer (pH 7.7) was determined by empirical inoculations of serial dilutions on *Nicotiana glutinosa* and *N. tabacum* 'White Burley'. Plants were inoculated on the first fully developed leaf from the top and each inoculated leaf was labelled.

Prior to the inoculations, all plants were tested for the absence of ToBRFV by RT-qPCR, and for pepino mosaic virus (PepMV) and tomato mosaic virus (ToMV) by double antibody sandwich (DAS)-ELISA using antisera from Prime Diagnostics according to standard methods.

2.2.2 | Symptom assessment and plant sampling

Starting 1 week after inoculation, all plants were visually assessed for the development of symptoms on a weekly basis up to a total of 2 months (data not presented).

To assess the development of virus infections, plants were sampled three times per week for the first 2 weeks, at three different positions: the top leaf, and the leaves above and below the inoculated leaf. From 3 weeks onwards, plants were sampled once a week from the top leaf, a leaf near a developing truss and a sepal of a developing fruit.

2.2.3 | ToBRFV screening

Plant samples were tested in duplicate with a standard DAS-ELISA protocol (Prime Diagnostics, https://www.wur.nl/en/show/Prime-Diagnostics-2.htm) using an antiserum developed for the detection of ToBRFV (Prime Diagnostics). Leaf samples and calyx samples (two pooled calyxes from two fruits per plant) were ground in sample extraction buffer (SEB; 1:2 weight to volume).

Total RNA was extracted from 100 µL of the SEB sample homogenates using the Qiagen Plant Mini kit. RT-qPCR was performed essentially as described in the protocol of The International Seed Health Initiative for Vegetable Seeds (ISHI-Veg; International Seed Federation, 2020) with two modifications. The qScript XLT 1-step RT-qPCR mix was used instead of the Ultraplex 1-step Toughmix (both Quantabio) and 2 µL of total RNA was used instead of 5 µL.

2.3 | OC trial

2.3.1 | Sample collection

Samples were taken from two tomato production locations infested with ToBRFV, in the winter of 2019 (Site 1) and 2021 (Site 2). Tomato plants with symptoms of ToBRFV infection were present at both sites. All tomato plants in both greenhouses were mature at time of sampling and previously infected with PepMV as a cross-protection measure for that virus. The samples and sampling strategies differed slightly per greenhouse but at both sites, strict hygienic precautions were taken to prevent contamination among sampled plant parts. Samples were taken from available plant parts and labelled according to their position on the plant (Figure 1).

Site 1

There were two sampled groups: symptomatic plants and asymptomatic plants. Symptomatic plants were sampled from the same row and showed clear symptoms associated with ToBRFV: chlorotic mottle and narrowing of the young leaves. The asymptomatic plants grew in the same row as the symptomatic plants but were sampled from plants at a reasonable distance, with multiple healthy looking plants in between. The sampled asymptomatic plants also stood several plants apart from each other.

Each group, consisting of either symptomatic or asymptomatic plants, included five plants and the following plant parts were sampled per plant in the following order: (part of the) lowest old leaf (old leaf 1), old leaf approximately 2 m from the ground (old leaf 2; highest reachable leaf), sepals from lowest truss, sepals from second lowest truss, young leaf from top of the plant (Figure 1). A total of 50 samples were taken.

Site 2

There were three sampled groups: symptomatic plants, asymptomatic plants from the same row as the symptomatic plants and asymptomatic plants from a healthy looking part of the cultivation, at least 30 rows (approx. 1.5 m between rows) away from the symptomatic rows.

Again, five plants were sampled per group, but two additional parts per plant were sampled: fruit from the lowest and the second lowest truss (see Figure 1). The sepals were sampled from the corresponding fruit. Otherwise, the samples were taken in the same way as at Site 1. Hence, seven samples were taken per plant, leading to a total of 105 samples.

The time of infection probably differed per greenhouse, due to the 'real world' conditions of dealing with active outbreaks, and the exact time of infection could not be determined. According to the grower, Site 1 was infected with ToBRFV for the first time, while Site 2 was sampled during a second infection. Despite strict hygiene measures during crop rotation at this site, after several weeks into the new cultivation, the plants of the new cultivation started to showed symptoms and ToBRFV was detected again. Although the source of the second infection remains uncertain, the sequences were similar to the prior infection (data not presented), supporting the report of re-infestation from the first infection.

For both sites, samples were stored at −80°C until tested.

2.3.2 | ToBRFV screening

RNA was extracted from samples and screened for ToBRFV at Naktuinbouw as described by van de Vossenberg et al. (2020). In brief, RNA was extracted from the various leaf parts using the Sbeadex Maxi Plant Kit (LCG Genomics) on the automated KingFisher Flex 96 platform (Thermo Fisher) and tested for presence of ToBRFV with the RT-qPCRs using primers/probe sets CaTa28 and CSP1325, described by ISHI-VEG (International Seed Federation, 2020). Testing was carried out on a CFX96 thermal cycler (Bio-Rad) according to the manufacturer's instructions. The average C_t values were recorded for each sample and compared. When the RT-qPCR produced a C_t value of ≤32, the virus was regarded as present; thresholds were set based on validation data generated by Naktuinbouw during test development (EPPO, 2022a) and in general use at the Netherlands Food and Consumer Product Safety Authority (NVWA), Netherlands.

Due to the widespread use of cross-protection strategies for management of pepino mosaic virus (PepMV, genus *Potexvirus*) all plants sampled for the outbreak comparison study were also previously treated with PepMV (data not presented). Co-infection with PepMV was not used in either inoculation experiment.

3 | RESULTS

3.1 | Inoculation experiment 1

3.1.1 | Overview of ToBRFV detection

Detection of virus from different plant parts with respect to time after inoculation is presented in Figure 2 and Table 1. In early infections, the virus was detected in leaves from the top of the plant 13–28 days postinoculation (DPI), in leaves from the middle of the plant approximately 2 weeks later, and in the lower leaves a further 2 weeks later. This timing was broadly similar between varieties tested. C_t values decreased more slowly in lower and middle leaves than upper leaves, indicating the virus concentration increased more slowly in these plant parts. Plants inoculated early in the growth cycle were highly susceptible to infection, with 15 of the 16 plants inoculated over the two early treatments becoming readily infected. The other remaining plant (spring crop/early inoculation; plant 'a') had some positive leaf detection results, but a different pattern of detection was observed; the first positive leaf was in the middle of the plant after 28 days and the next positive leaf was in the lower part of the plant at 63 days. This indicates that the plant may have become infected later in the growth cycle and not during the initial trial inoculation.

In both spring and winter 'late inoculation' trials, the movement and consequent detection of the virus throughout the plant appeared to be highly erratic, as shown in Figure 2. This erratic distribution and inconsistency of virus concentration had a confounding impact on the interpretation of results for the determination of positive and negative plants in the trial as a whole. Overall, fewer plants in the late inoculation treatments became infected, with 7 out of 16 plants having results on multiple occasions indicating some target amplification, but over the $C_t < 32$ threshold. In one case (spring crop, late infection; plant 'd') the pattern of infection was similar to that observed in early infections, although infection developed more slowly, with the earliest infected leaf in that plant detected at 49 DPI. In another case, (spring crop, late infection; plant 'e') virus detection was consistently strong in the top of the plant at every sample date from 28 DPI (C_t 7–19). However, the rest

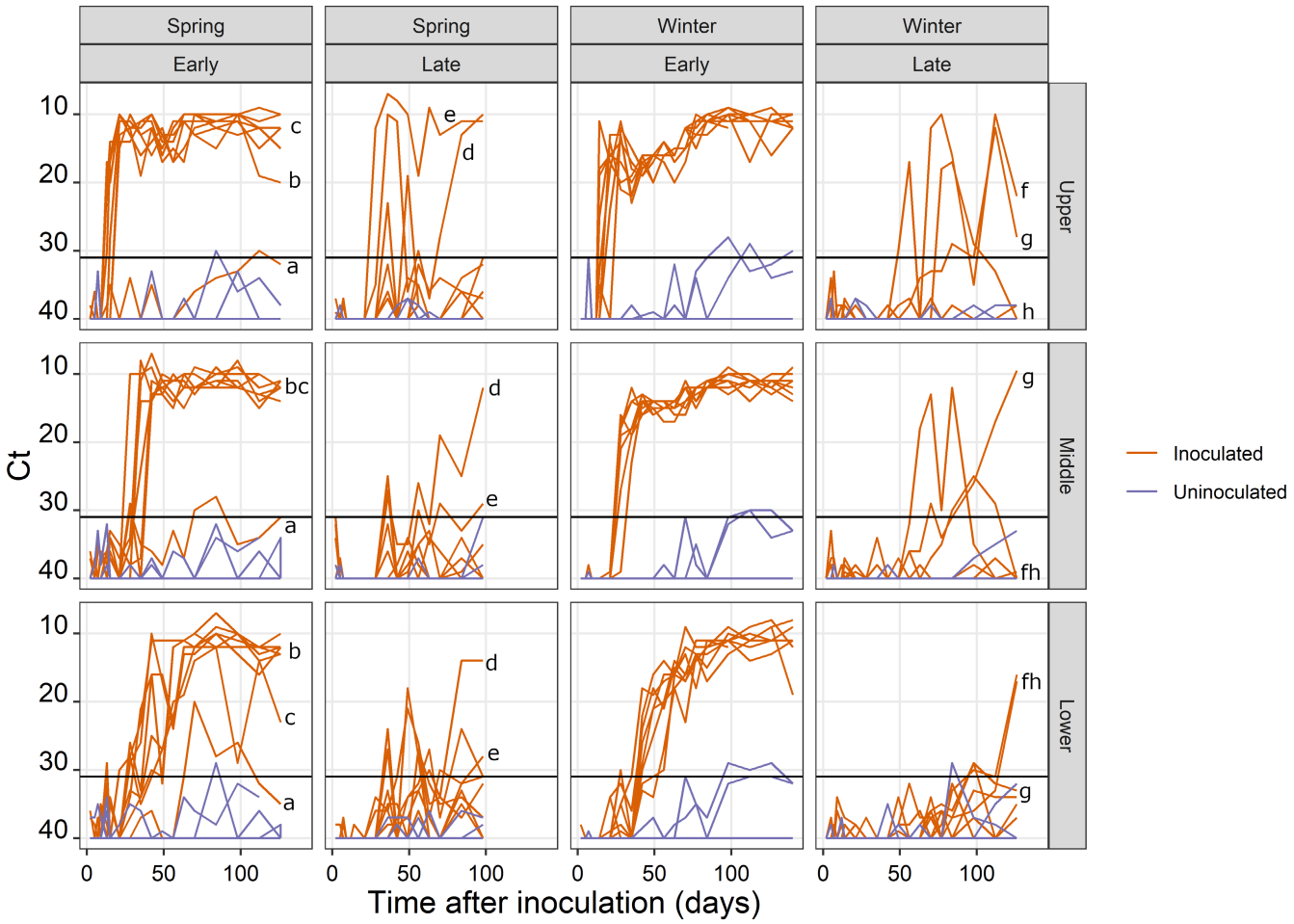

FIGURE 2 Detection of tomato brown rugose fruit virus in tomato plants by reverse transcription-quantitative real–time PCR; inoculation experiment 1. Graphs show cycle threshold values (C_t) of inoculated plants (orange) and uninoculated controls (purple). Individual plants that gave anomalous results to the general trend in the specific trial are labelled a–c (spring/early); d and e (spring/late); f–h (winter/late).

of the samples from that plant tested negative (C_t undetermined) for a further 4 weeks. Plants 'f' to 'h', in the late infected winter crop highlight the erratic nature of virus development in the late infected plants.

Over the course of the four infection treatments, over 1600 real-time PCR tests were carried out; therefore, full results from the trial are presented in the supporting information. Leaf sampling results are included in File S1, sepals and fruit are in File S2. In each case these are presented as RT-qPCR C_t values without any form of interpretation as to positive/negative. For the purposes of further analysis, results presented below were also interpreted as either positive (≤32) or negative. This application of a 'cut off' or 'diagnostic threshold' reflects the current practices employed in many laboratories routinely testing for ToBRFV using the ISF/ISHI-Veg protocol (International Seed Federation, 2020) or the EPPO protocol (EPPO, 2022a). The noninfected 'healthy' controls used in the experiments showed some amplification indicating potential virus presence in these plants, but results were erratic indicating these plants were probably contaminated by environmental residues of ToBRFV and not infected (File S1).

3.1.2 | Timing of detection from sepals and fruit compared to leaves

Results from the sepal and fruit testing are presented in Table 1. In early inoculated crops, sepals and fruit were not present until several weeks after inoculation, and these were sampled only once mature fruit were present. Consequently, virus was consistently detected earlier in the leaf samples than sepals and fruit, and generally in the upper leaves first. As soon as sepals and ripe fruit were available for testing, virus was consistently detected in these plant parts.

In late inoculated treatments, the sepals and fruit were generally found to be positive earlier than leaf samples. In one exception to this pattern, a spring crop/late inoculated plant was detected with a borderline positive result ($C_t = 31$) in a leaf from the middle of the plant 2 DPI, 12 days earlier than detection from fruit and 19 days earlier than detection from sepals in the earliest virus detections in that treatment. However, no further virus was detected in leaves from this plant until 36 DPI, almost 5 weeks later. By comparison in the same plant, the sepals and fruit were

TABLE 1 First detection by reverse transcription-quantitative PCR of ToBRFV in different parts (leaf, sepal and fruit) of tomato plants, and region of plant from which samples were taken (days after inoculation).

Infection time	Crop	Sample site	Leaf	Sepal	Fruit
Early	Spring	Upper	13	70[a]	126[a]
Early	Spring	Middle	28	63[a]	63[a]
Early	Spring	Lower	13	56[a]	56[a]
Early	Winter	Upper	14	77[a]	112[a]
Early	Winter	Middle	28	77[a]	77[a]
Early	Winter	Lower	28	77[a]	77[a]
Late	Spring	Upper	28	21	21
Late	Spring	Middle	2[b]	21	14
Late	Spring	Lower	36	14	21
Late	Winter	Upper	49	35	na
Late	Winter	Middle	63	35	35
Late	Winter	Lower	98	14	35

Note: Detection determined as days after inoculation at which samples had a $C_t < 32$.

Abbreviation: na, no detection.

[a]In early infected crops, sepals and fruit were tested at the earliest time, as soon as fruits were ripe.

[b]Individual plant result on the borderline of positive/inconclusive, virus was not detected again in this plant until 36 days after inoculation.

both strongly positive at 21 DPI (C_t of 13 and 12, respectively). In both the winter and spring inoculations, virus was detected from the sepals in the lower part of the plant earlier than from the middle and upper sepal samples. Further analysis was carried out on these data to estimate the probability of detecting a positive leaf, if sampled at random, with respect to different times after inoculation (File S3).

3.2 | Inoculation experiment 2

3.2.1 | Symptom assessment

All inoculated plants of both cultivars Brioso and Merlice started to show ToBRFV symptoms 1 week after inoculation. Symptoms consisted of leaf bubbling, developing into clear mosaic and nettle head symptoms with occasional necrotic spots in cultivar Brioso (data not presented).

3.2.2 | Timing of detection from leaves by DAS-ELISA

ToBRFV could be detected by DAS-ELISA in the top leaves of all the plants from both cultivars at 4 DPI (Table 2). At 7 DPI, ToBRFV could also be detected in the leaves just above the inoculated leaf in nearly all plants. At 21 DPI, although the virus still could not be reliably detected in leaves beneath the inoculated leaf in cultivar Merlice, it could be detected in some leaves beneath the point of inoculation of cultivar Brioso.

3.2.3 | Comparison of earliest detection by DAS-ELISA versus RT-qPCR

A random selection of plant samples was also tested by RT-qPCR (Table 3). This not only confirmed the presence of ToBRFV in all DAS-ELISA positive samples but also showed that ToBRFV could be detected in the samples of plants that did not test positive by DAS-ELISA at 4 DPI. At 2 DPI, ToBRFV was detected by RT-qPCR in one sample of cv. Merlice (Day 2, leaf b) and multiple samples of Brioso. This earlier detection may be a consequence of the comparative difference in limits of detection between ELISA and RT-qPCR.

From 28 DPI, sepals of developing fruits instead of leaves below the inoculated leaf were tested by both DAS-ELISA and RT-qPCR. For all plants these samples tested positive by both methods (data not presented).

3.3 | Outbreak sampling comparison

3.3.1 | Site 1

As both primer/probe sets used gave comparable detection, only those from CSP1325 are presented here; a full breakdown of outbreak sampling data is presented in File S4. At Site 1 (Figure 3), ToBRFV was detected in symptomatic plants, with C_t values being lowest in and similar for sepals and top leaves. In the older leaves (Old leaf 1 and 2), average C_t values were significantly higher with C_t values around and above the >32 C_t threshold; C_t values of individual samples varied between 25 and 36. In the asymptomatic plants, ToBRFV C_t values of individual samples varied between 29 and 40, with the average C_t values per plant part greater than C_t 35.

3.3.2 | Site 2

At Site 2 (Figure 4) ToBRFV was detected by RT-qPCR in both symptomatic and asymptomatic plants. In the symptomatic plants, C_t values were lowest and similar for sepals, fruits and leaves from the top. The C_t values were higher in older leaves. However, at Site 2, the differences in C_t values among the plant parts were less clear than at Site 1. In both groups of asymptomatic plants, that is, plants from the same rows and from rows at a distance from symptomatic plants, ToBRFV was detected in most of the samples and there were no significantly different C_t values among the various plant parts. These results differed from those obtained at Site 1.

TABLE 2 Double antibody sandwich-ELISA detection of ToBRFV in tomato leaf samples at different time points after inoculation (inoculation experiment 2).

Sample			No. of days after inoculation						
Cultivar	Plant	Leaf[a]	2	4	7	9	11	14	21
Merlice	1	a	−	+++	+++	+++	+++	+++	+++
		b	−	++	+++	+++	+++	+++	+++
		c	−	−	−	−	−	−	−
	2	a	−	++	+++	+++	+++	+++	+++
		b	−	−	+++	+++	+++	+++	+++
		c	−	−	−	−	−	−	+
	3	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	−	−	−	−	+
		c	−	−	−	−	−	−	−
	4	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	+++	−	+++	±	+++
		c	−	−	−	−	−	−	−
	5	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	−	−	±	−	−
		c	−	−	−	−	−	−	−
	6	a	−	+++	+++	+++	+++	+++	+++
		b	−	+++	+++	+++	+++	+++	+++
		c	−	−	−	−	−	−	−
Brioso	1	a	−	+++	+++	+++	+++	+++	+++
		b	−	+	+++	+++	+++	+++	+++
		c	−	−	−	+++	−	−	−
	2	a	−	+++	+++	+++	+++	+++	+++
		b	−	±	+++	+++	+++	+++	+++
		c	−	−	−	−	−	−	±
	3	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	+++	+++	+++	+++	+++
		c	−	−	−	−	−	−	−
	4	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	+++	+++	+++	+++	+++
		c	−	−	−	−	−	−	+++
	5	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	+++	+++	+++	+++	+++
		c	±	−	−	−	−	−	+
	6	a	−	+++	+++	+++	+++	+++	+++
		b	−	−	+++	+++	+++	+++	+++
		c	−	−	±	−	−	−	−

Note: Assessment after 1 h substrate incubation. ELISA results are presented as A_{405} in excess of the negative threshold. ELISA result: +++, $A_{405} > 1$; ++, $0.5 < A_{405} > 1$; +, $0.25 < A_{405} > 0.5$; ±, $0.1 < A_{405} > 0.25$; −, $A_{405} < 0.1$.

[a]Position of leaf: (a) top leaf, (b) leaf just above the point of inoculation, (c) leaf below point of inoculation.

4 | DISCUSSION

As early as 1934, the movement of viruses had been studied in tomato using the related tobamovirus, TMV (Samuel, 1934). This prior study was carried out decades before serological or molecular diagnostic tools were available and used bioassay (sap inoculation to test plants) for confirmation of virus entering leaf tissue and different plant parts. The results from both early inoculation experiments in the current study show a similar pattern of movement, where the virus is detectable in the upper plant first and then spreads back

TABLE 3 Comparison of ToBRFV detection by reverse transcription-quantitative real–time PCR (RT-qPCR) with different primers/probes and by double antibody sandwich (DAS)-ELISA in samples from different leaves of tomato plants at different time points after inoculation.

Sample				ToBRFV DAS ELISA[b]	ToBRFV-CaTa28 RT-qPCR[c]	ToBRFV CSP1325 RT-qPCR[d]	BaCV RT-qPCR[e]
Cultivar	Plant	Days after inoculation	Leaf[a]				
Merlice	1	2	a	0.00	37.1	37.1	24.8
			b	0.00	36.2	36.7	25.1
			c	0.00	35.4	36.0	25.2
	2	2	a	0.00	30.2	31.7	24.2
			b	0.00	36.9	37.2	24.6
			c	0.00	32.2	34.1	24.4
	1	4	a	2.10	14.3	16.0	24.3
			b	0.37	20.8	23.7	27.1
			c	0.00	31.8	33.3	26.4
	2	4	a	1.12	17.6	19.8	24.9
			b	0.00	27.9	29.2	26.0
			c	0.00	32.5	33.5	25.2
	4	14	b	0.16	21.5	23.4	24.9
	5	11	b	0.09	23.0	25.1	25.2
Brioso	1	2	a	0.00	29.6	29.9	24.3
			b	0.00	31.0	32.4	23.6
			c	0.00	29.7	31.0	23.6
	2	2	a	0.00	24.5	25.9	23.0
			b	0.00	25.7	27.2	24.6
			c	0.00	28.1	29.7	24.6
	1	4	a	2.39	10.5	14.3	24.8
			b	0.48	20.2	22.3	24.0
			c	0.00	32.0	32.3	23.7
	2	4	a	2.25	13.1	15.0	25.0
			b	0.19	22.4	24.1	25.2
			c	0.00	33.8	34.5	24.4

Abbreviation: BaCV, Bacopa chlorosis virus used as an internal control.

[a]Position of sample: (a) top leaf, (b) leaf just above the point of inoculation, (c) leaf below point of inoculation.

[b]ELISA results are presented as A_{405} in excess of the negative control threshold. A_{405} 1 h after addition of substrate.

[c]C_t, probe Ca Ta 28 fluorescently labelled with FAM.

[d]C_t, probe CSP1325 fluorescently labelled with VIC.

[e]C_t, probe for BaCV labelled with Texas Red.

down through the plant. Comparing the results from both early inoculation experiments in this study with those from the 1934 study suggests that, in theory, there may be a difference of approximately 2–8 days between the virus being present in the upper leaf tissue and it being detected by serological or molecular methods. This time difference for detection appears to be influenced by the age of the plant at the time of infection. The key difference between the two inoculation experiments reported here was the age at which plants were inoculated. In IE2 plants were approximately 4 weeks from sowing, while in IE1, 'early inoculation' was around 8 weeks after sowing. The physiological changes in the plant as it ages appear to affect the speed of movement of the virus and/or its replication efficiency. The results from Samuel indicate the virus moves to the root then quickly to the upper part of the plant and then back down to the lower leaves to give total systemic infection after approximately 25 days. In IE1, first detection of virus in lower leaves from early infection appears to occur between 13 and 28 days. In IE2, this first detection appears at 21 days. This does not seem to be influenced by the age of the plants at time of infection in the different experiments. In another similarity to the Samuel (1934) study, infection in older plants showed a more erratic distribution. As noted by the Samuel study, "...the presence of a developing fruit truss a few nodes above the insertion may sometimes exert a pull...", and this appears to be supported by the results from the current study. This phenomenon has since been referred to as the source–sink hypothesis of viral movement and accumulation (Hull, 2014). Nearly 90 years after

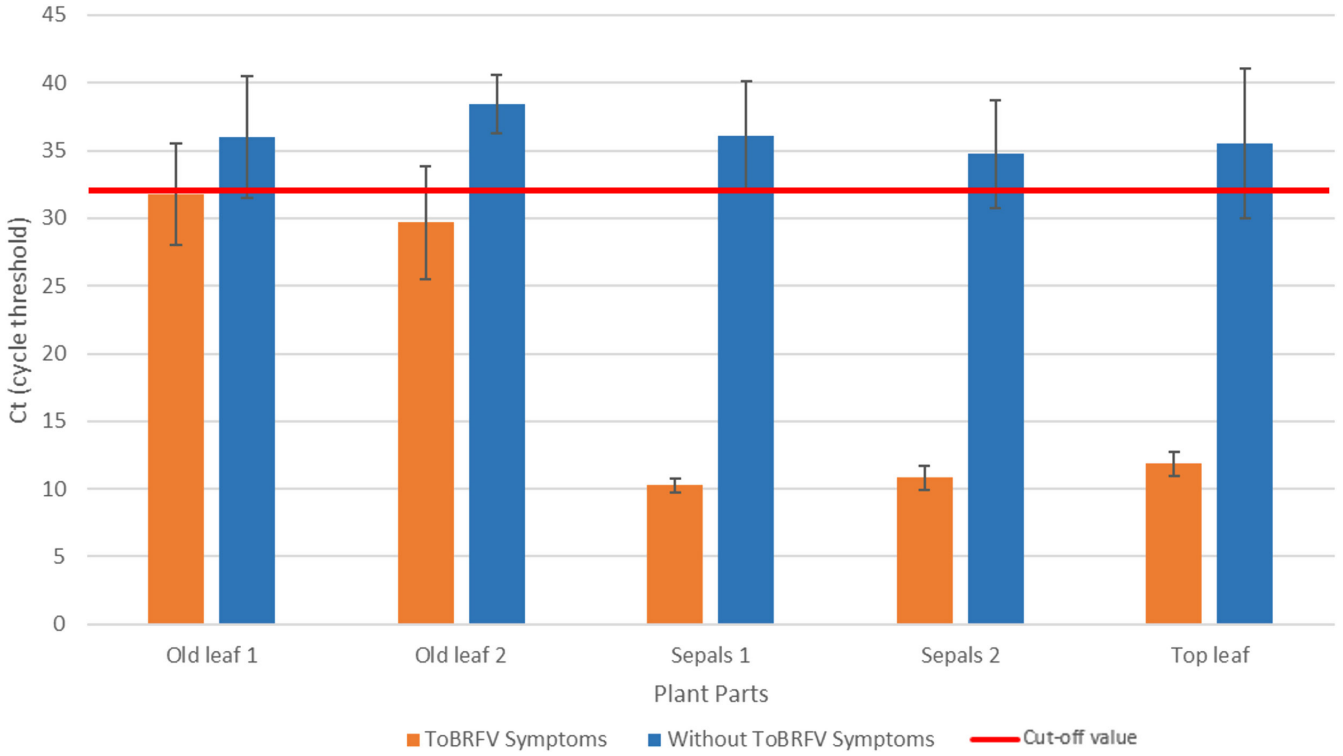

FIGURE 3 Detection of tomato brown rugose fruit virus in different parts of tomato plants by reverse transcription-quantitative real–time PCR; outbreak comparison (OC) trial, Site 1. Symptomatic plants (orange) were sampled from the same row and showed clear symptoms associated with ToBRFV. Asymptomatic plants (blue) grew in the same row as the symptomatic plants but were sampled from plants at a reasonable distance, with multiple healthy looking plants in between. Average C_t values per plant part of symptomatic and asymptomatic plants are shown. Bars represent the standard deviation.

Samuel (1934) performed his experiments using different diagnostic approaches, similar patterns of movement in both young and old infected plants have been observed. Additionally, infection in older plants showed a more erratic distribution of virus, supporting the earlier conclusions on a source–sink movement of virus. The results from the OC trial show that the virus can be reliably detected in sepals and fruits. This also corresponds with the Samuel study indicating these sinks are the most suitable sampling material for the detection of viruses.

Test validation studies tend to focus on the analytical sensitivity and analytical specificity of a test, determining the limit of detection and characteristics of the test (Chabirand et al., 2016; Roenhorst et al., 2018). Currently, many laboratories implement diagnostic cut–offs, that is, a decision threshold dependent on validation data supporting a positive/negative inference. However, for applications in surveillance, ELISA is recognized to give comparatively less sensitive detection than RT-qPCR; despite this comparative difference, as with other methods with restricted sensitivity, ELISA can still be applied to surveillance testing if the appropriate validation is carried out to include optimal sampling and bulking strategies. With the reliance on diagnostic surveillance to demonstrate absence of the virus, it is critical to devise sampling strategies to maximize the chance of early detection of the virus. Therefore, there is a need to understand better the reliability of virus detection with reference to infection dynamics of different crops and viral genera within the plant.

The aim of the inoculation experiments was to try and replicate commercial growing conditions as closely as possible within a strict quarantine environment, resulting in limitations to the size and scope of experiments that could be carried. This aim dictated the parameters of the experimental set–up such as variety choice, growing conditions and inoculation timing. The varieties used in the studies are varieties grown widely in north-west European protected tomato production and represent different fruiting types. Inoculation times were chosen to simulate different infection scenarios, 'early infection' occurring as plants are moved from the propagation nursery to the production facility, and 'late infection' representing infection entering a fruit production facility in the middle of the growing season. In the late infection treatments (mature plants), the sepals and fruit were consistently clearly determined as virus positive before the virus could be reliably detected from young or older leaf tissue.

In two of the studies reported here (IE1 and OC), diagnostic testing was carried out by RT-qPCR, using primer/probe sets listed in the EPPO diagnostic standard for detection of ToBRFV (EPPO, 2022a), validated through the Euphresco Valitest project (Luigi et al., 2022), and in compliance with the EU emergency regulations. In the inoculation trial, the primers were those from Menzel and Winter (2021), and in the OC trial, the CSP 1325 primers developed by the International Seed Federation were used (International Seed Federation, 2020). Although different primer sets were used

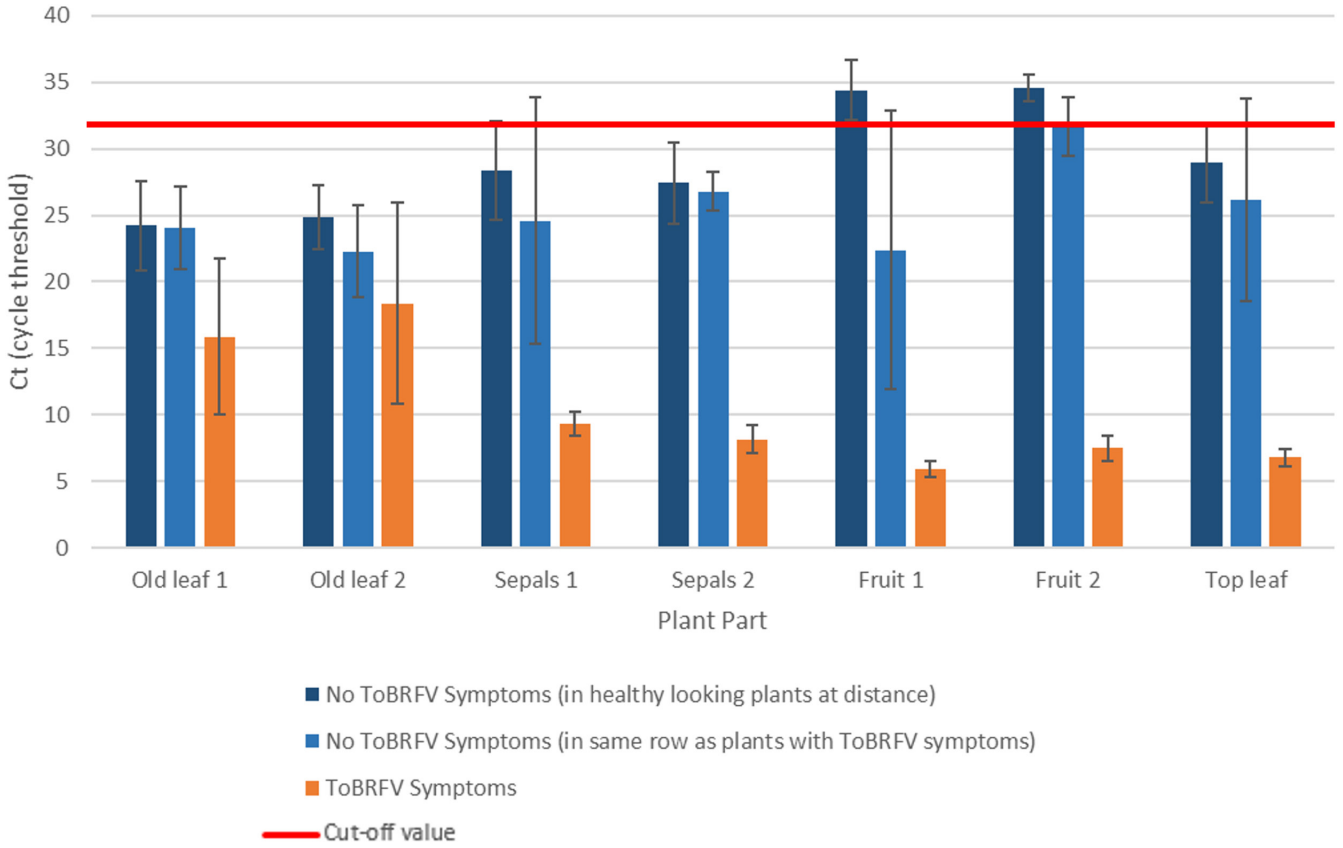

FIGURE 4 Detection of tomato brown rugose fruit virus in different parts of tomato plants by reverse transcription-quantitative real–time PCR; outbreak comparison (OC) trial, Site 2. There were three sampled groups: symptomatic plants (orange), asymptomatic plants from the same row as the symptomatic plants (light blue), and asymptomatic plants from a healthy looking part of the cultivation, at least 30 rows (approx. 1.5 m between rows) away from the symptomatic rows (dark blue). Average C_t values per plant part of symptomatic and asymptomatic plants are shown. Bars shown represent the standard deviation.

for the diagnostic screening, this gives a better representation of the actual 'real world' situation where regulatory laboratories have a selection of diagnostic tests they can use. In conclusion, based on the results of the two inoculation experiments, it is likely that the greatest influence on detection, related to rate of translocation and distribution of the virus in the plant, is dependent on plant age at inoculation rather than the detection method applied. Based on the results presented here, ELISA may be an equally appropriate method for use in supporting surveillance diagnostics; however, other factors such as relative analytical sensitivity, specificity and bulking rates may need to be taken into consideration.

It should be noted that the OC trial samples were taken from 'live' outbreaks and so the precise starting point of infection at both sites could not be determined; therefore, it is unknown how many days after infection the crops were sampled. Two tomato production sites were sampled for the OC study. According to the grower, Site 1 was infested with ToBRFV for the first time, while Site 2 struggled with a recurring (secondary) infestation (NPPO-NL). Despite strict hygiene measures during crop rotation at Site 2, the plants started to show symptoms several weeks after the new cultivation and ToBRFV was detected again. Although the source of the second infection remains uncertain, the ToBRFV sequences of the primary and secondary infections were highly similar indicating a re-infestation. Also, it remains uncertain if all the plants from the symptomless rows were actually infected with ToBRFV or not at the time of sampling, or if some of these detections were environmental residues carried over from the prior infection. Interestingly, a difference was observed in the level of detection of the virus in the older leaves between the two sites: at Site 1, the virus was just or not detectable in the old leaves of asymptomatic plants, while at Site 2, the virus was clearly detected in the older leaves of asymptomatic plants at C_t levels lower than in young leaves, sepals and fruits. This raises the question as to whether this detection was from an active infection or from environmental contamination with pollen, dust, and so on from highly infected plants in close proximity.

The observation of symptoms in the OC samples indicates that the presence of symptoms such as bubbling, chlorosis, mosaics and narrowing of leaves are generally a good indication of the presence of virus infection, although this is not diagnostic specifically for ToBRFV. However, these data also show that the absence of symptoms is not an effective way to assess freedom from infection; in both the IE1 and the OC study, there was evidence of virus presence where infection was not apparent and even where plants had been separated either through screening and separation of irrigation systems (IE1) or

through physical distance from point of infection (e.g., OC, Site 2). Outbreaks of contact-transmitted viruses are a dynamic situation with virus present and replicating in asymptomatic plants for a period before symptoms are evident. Panno et al. (2020) noted transmission of ToBRFV to previously healthy plants that had not had physical contact with infected plants. This was thought to be the result of bumblebee-mediated transmission in line with the demonstration of this transmission route by Levitzky et al. (2019). However, in the inoculation study, the 'healthy' control plants all became infected during the course of the trial despite fastidious application of contamination controls and no use of bumblebee pollination; these plants were screened from the infected plants and working patterns ensured these plants were handled first. In the 'late' inoculation plants there was also potential on-plant contamination by detectable environmental residues of virus throughout the experiment where plants that were deemed to be 'uninfected' tested positive inconsistently. The results for the 'distant' asymptomatic plants in the OC trial also appear to show the presence of similar environmental residues on apparently healthy plants with consistent virus detection on all plant parts. Swab testing of glasshouses with ToBRFV outbreaks can give 'positive' results from surfaces that have not had direct contact (Loh et al., 2022). The precise nature of these sources of environmental residues are not yet known but may include 'dust', consisting of broken trichomes and pollen, from infected plants, or may also be the result of contaminated water vapour carrying virus. The presence of tobamoviruses in water vapour (fog and clouds) and in the wider environment has been previously reported (Castello et al., 1995; Fillhart et al., 1998). The phytosanitary relevance and relative importance of this as a transmission risk in a protected cropping environment has not been determined. However, the presence of such residues, and the long-term survival of detectable virus on surfaces (Loh et al., 2022; Skelton et al., 2021) may present a 'diagnostic risk' resulting in erroneous reporting of crop infection in outbreak glasshouses in successive seasons following post-outbreak clean-up. The importance of environmental residues as both a transmission risk and a diagnostic risk need to be investigated further.

The inoculation studies reported here indicate that the key influence on movement of the virus within the plant was the age of the plant at time of inoculation rather than other factors such as cultivar or growing conditions. Early infection resulted in a predictable pattern of virus development in the plant, whereas infection of mature plants resulted in more erratic development of the virus infection, supporting the results reported by Samuel (1934). These results, when combined with the results from testing different plant parts from an outbreak of unknown infection timing, indicate that sampling regimes for surveillance must be flexible to account for different stages of the cropping cycle. To give the greatest chance of ToBRFV detection, recommendations for surveillance sampling arising from this work are that (1) sampling from young crops (before truss set) should focus on taking young, actively growing leaves, and (2) following truss formation, sampling should focus on sepals and fruit as well as young actively growing leaves. It is important to note that these studies focus on hydroponic protected cropping systems, as used in north-west Europe, and the relevance for field-grown tomatoes should be evaluated.

To formulate practical sampling information for feasible surveillance strategies, it is pivotal to gather data from 'real world' conditions to substantiate the experimental data. In both studies reported here, the virus was reliably detected in the sepals, fruits and young leaf tissue, while in older leaves the detection was inconsistent. It is also key to note that due to the source–sink hypothesis, more mature plants may not be as susceptible to fully systemic infection as young plants, with a much lower proportion of plants developing infection, and infection not developing in all plant parts. The phenomenon, termed 'mature plant resistance', has been noted in other crops, mainly in relation to insect-transmitted viruses of potato and cereal crops (Gibson, 1991; Lindblad & Sigvald, 2004; Sigvald, 1985). Further work on a larger scale is needed to confirm if this phenomenon is at play in the tomato–tobamovirus pathosystem, but the results presented here suggest systemic virus infection develops more slowly in mature plants. This may indicate that the risk of virus infections in crops diminishes with the age of the crop. Therefore, efforts should be focused on maximizing biosecurity and crop hygiene on plants entering the glasshouse and in the younger growth stages. The applicability of these data to 'real world' situations should be interpreted with caution, given the low numbers of plants tested and the 'mock' conditions that could not accurately replicate a commercial glasshouse, as well as the high levels of inoculum compared to a real outbreak, and the obvious stress plants were under once inoculated within this trial. Additionally, there are unknowns regarding infection dynamics of plants infected at time points not covered within this inoculation trial. However, this is the first study to investigate the chance of detecting the virus with respect to the within–plant spread of ToBRFV. This gives a strong indication that sampling regimes for ToBRFV surveillance should be altered to account for the results presented here, with a focus towards sepal sampling. The results of these experiments should be considered when designing surveillance strategies for ToBRFV.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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