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# Analysis of the Spatial Dispersion of Tomato Brown Rugose Fruit Virus on Surfaces in a Commercial Tomato Production Site

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**Abstract:** The tomato brown rugose fruit virus (ToBRFV) causes severe damage to tomato cultivars and has international economic importance. The harmful tobamovirus is easily mechanically transmissible and highly stable. An ongoing cultivation of infected tomato plants may lead to the spread of ToBRFV in and around the production area of the infested tomato farm. We conducted a study in which we collected a representative number of swab samples from various inanimate surfaces in greenhouses, packaging halls, and shared and private accommodations. In addition, numerous fabrics, such as outer clothing, bed linen, and items used by greenhouse workers, were tested. The infectivity of ToBRFV-contaminated surfaces was tested in bioassays using *Nicotiana tabacum* cv. Xanthi NN and confirmed using DAS-ELISA. The proportion of ToBRFV-contaminated surfaces varied among locations, from 48.7% in greenhouses to 0% in offices with limited access to staff. Samples from shared accommodation and private accommodation were 18.4% and 3.6% ToBRFV positive, respectively. Clothing and protective items were found to be highly contaminated with ToBRFV, and even around the sleeping area, infective ToBRFV was detected in a few apartments. This study provides evidence for the first time on how and where infectious ToBRFV can be spread by humans beyond the production area. To avoid further dissemination, strict hygiene protocols are required to interrupt transmission routes.

**Keywords:** bioassay; accommodation; packing hall; greenhouse; vehicle; fabric; protective items; contamination



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## 1. Introduction

Currently, the tomato brown rugose fruit virus (ToBRFV) is considered the greatest threat to tomato production worldwide [1]. During the first outbreak in Germany in 2018 [2], several tomato greenhouses on different farms were affected. Since then, the virus has been repeatedly detected in German greenhouses, outdoors under polytunnels, in private gardens, and on seeds [3], resulting in well over 50% loss of marketable tomatoes (personal communication).

In the last three decades, viral diseases such as pepino mosaic virus (PepMV) [4], tomato torrado virus (ToTV) [5], and tomato mottle mosaic [6] have emerged worldwide, posing a threat to tomato production [7]. Yield losses have been measured and estimated for individual viruses [8–10] and are particularly large when plants are infected early in development. In 2016, a new tobamovirus, tomato brown rugose fruit virus, was first identified [11]. Since the first outbreaks in Israel [12] and Jordan [11], ToBRFV has become a significant pathogen of tomato plants, causing devastating disease outbreaks and resulting in serious yield losses in many countries [7,13]. It was detected in 22 European countries, including the four largest producers, Spain, Italy, Poland, and Portugal [14–16]; Egypt in Africa [17]; China, Iran, Israel, Jordan, Lebanon, Saudi Arabia, Syria, and Turkey, Uzbekistan

in Asia [11,12,16,18–22]; and Canada, Mexico, and the US in North America [23–25]. Although the pathogen has been detected on four continents to date, genetic diversity among isolates is low [26].

The tomato (*Solanum lycopersicum* L.) is a major food crop and an important economic contributor to the primary sector, accounting for a global harvest of approximately 184.786 million metric tons and an estimated gross production value of USD 102.622 billion in 2020 [27]. During cultivation or post-harvest storage, tomatoes are susceptible to more than 200 yield-reducing diseases [28]. These diseases are caused by a variety of pathogenic fungi, bacteria, viruses, and nematodes, and they directly or indirectly result in losses in tomato production. In addition to the high water requirements of tomatoes, viral diseases, which cannot be curatively controlled with either pesticides or biological control agents, are an important factor limiting production worldwide.

ToBRFV infection in tomato plants can result in a range of symptoms, from mild to very severe. Fruits often display undesirable yellow and orange marbling or namesake dark "brown rugose" spots. These discolorations make fruits unmarketable [2]. In addition, González-Concha and colleagues recorded a 25 to 40% reduction in average fruit weight in greenhouse tomatoes [13]. Infection trials show ToBRFV-related yield reductions of 19 to 55%, depending on climate and cultivation methods [29]. Interestingly, these reductions were independent of the presence of the *Tm-2* resistance gene. The resistance gene *Tm-2*, which was introduced into cultivated tomatoes, confers strong or near complete resistance against TMV, tomato mosaic virus (ToMV), and tomato mottle mosaic virus (ToMMV) [12,30]. This gene has two resistant alleles: *Tm-2* and *Tm-2*$^2$ [31], with the latter becoming the most widely used ToMV resistance in breeding programs, and thus most, if not all, commercial tomato hybrids have this resistance today. Although the resistance gene *Tm-2*$^2$ was effective for over 40 years, ToBRFV appears to have overcome this genetic resistance to tobamoviruses [12,29,30].

The pandemic spread of ToBRFV in tomato crops was caused by the breaking of the resistance gene *Tm-2*$^2$ and the global trade of plants and seeds, which was enhanced by the easy mechanical transmissibility paired with the high stability of the virions. Tobamovirus virions are rod-shaped and viable in the environment outside the host plant for a long period of time [32,33]. Tobamoviruses such as ToBRFV are transmitted primarily mechanically through infected plants, plant debris, contaminated soil [34], tools [1,7], and items, for example, by farm workers handling both infected and non-infected plants [35,36]. The circulation of nutrient solutions contaminated with viruses is another dissemination path [37]. Klap and colleagues showed that damaged fruits can be an effective inoculum for virus transmission [38]. Tomato pollinators such as bumble bees (*Bombus terrestris*) may also transmit ToBRFV mechanically during flower pollination [39]. However, as with other tobamoviruses, virus vectors are unknown. In addition, low seed-to-seedling transmission rates, ranging from 0.08% to 2.8%, have been demonstrated in previous studies [40,41]. The global movement of the virus via contaminated seed appears to be the only credible explanation for the observed intercontinental movement of ToBRFV. Thus, in many countries, the testing of seed or parent plants is required as part of emergency measures to prevent ToBRFV entry through the importation and movement of ToBRFV host seed, tomato, and pepper. In the event of an outbreak of ToBRFV, emergency measures such as the destruction of infected plants are mandatory to stop the establishment and spread of the virus. These official eradication measures based on (EU) 2020/1191 were revised in the EU in 2021. Producers of tomato fruits can therefore continue cultivating until the end of the growing season despite the detection of a ToBRFV infestation.

This will likely lead to a significant spread of the virus throughout the farm and possibly beyond. Contamination with ToBRFV will not only affect the direct production area but might also spread to parts of the farm not related to tomato production. In parallel, the new Implementing Regulation (EU) 2021/1809 also requires specific hygiene measures for personnel, production site structures, tools and machinery, materials, and means of transport. In light of this changing regulatory framework, sanitation and disinfection measures also need to be adapted if ToBRFV spreads outside the production area. The

extent to which the stable tobamovirus can adhere to inanimate surfaces, survive on them, spread, and, upon contact with a suitable host plant, cause infection, remains to be investigated. Using these insights, hygiene management can be specified individually for each farm in terms of both preventing the introduction of disease and, following an outbreak, preventing the further spread of the virus. A study of the tobamovirus cucumber green mottle mosaic virus (CGMMV) on greenhouse surfaces showed that huge parts of the infested greenhouse were contaminated with CGMMV [42]. Based on a recent ToBRFV outbreak on a farm, our study provides additional insights into the carryover and persistence of ToBRFV in farm areas such as the living quarters.

Sequence data from ToBRFV outbreaks in the Netherlands indicate that in some farms, despite intensive cleaning and disinfection measures after an outbreak, the virus was not successfully eradicated and reappeared in the following crop cycle [43]. In this context, it is important to determine ToBRFV persistence on different inanimate surfaces in a farm in order to adapt eradication measures accordingly.

In order to obtain an initial impression of the extent of ToBRFV contamination on a practical farm, standardized sampling of a ToBRFV-infested farm was carried out by taking hundreds of swab samples from surfaces in the production area, packaging, and the living quarters of seasonal workers. Surfaces made of plastic, stainless steel, and fabric were investigated. We explored the following questions regarding ToBRFV: (i) Where does it adhere, (ii) to what type of surfaces, (iii) at what frequency, and, most importantly, (iv) is it still infectious?

## 2. Materials and Methods

### *2.1. Demarcated Production Site*

This sampling study was conducted on a ToBRFV-infested tomato farm in Germany in 2022. The farm manager reported that in spring 2022, unexplained symptoms appeared in tomato and pepper varieties in his greenhouses. In mid-July 2022, ToBRFV was detected following official testing for ToBRFV by the responsible plant protection authority. At that time, about 30% of his tomato plants were already affected and showed symptoms such as uneven ripening of young fruits, orange fruits not turning red, and a reduced number of fruits per branch. On this farm, two samplings were carried out by the authors of this study. At the time of the first sampling in autumn 2022, according to the farm manager, a large part of his 1.67 ha greenhouse production area, which was mainly used for growing the commercial tomato cultivar "Mecano F1," had already been affected, and some greenhouse areas had already been cleared at that time. At that time, tomato fruit production was still carried out by about 10 employees.

The second sampling also took place in autumn 2022. At this point, the greenhouses had already been cleared, and all employees had left the company. During this second sampling, particular focus was placed on the now-unoccupied private accommodations of the workers. As the farm has no hygiene gates or other sanitary facilities in the production area, a high risk of ToBRFV carryover by employees was already assumed in advance of the sampling.

### *2.2. Sampling in a Demarcated Tomato Farm*

In order to investigate the objective of contamination severity and the extent of ToBRFV carryover throughout the farm, sampling of outer clothing, gloves, shoe covers, and parts of the bedding was conducted, and swab samples of potentially ToBRFV-contaminated surfaces were also taken. The focus of this study was not the spread of ToBRFV in the crops, but rather the carryover on inanimate objects in and around the production area. Table 1 gives an overview of the number of samples taken and an insight into which objects and surfaces were sampled.

**Table 1.** Overview of taken samples in a tomato brown rugose fruit virus (ToBRFV) infested farm.

| Type of Sample | Location | Sampled Object | No. of Samples |
|---|---|---|---|
| Carrier | Greenhouses | T-shirts, gloves, shoe covers | 178 |
| | Private accommodation | mattresses, bed cover, pillowcase | 81 |
| Swab | Greenhouses | Concrete floor, foil, foot mats, rails, pipes, tubes, etc. | 78 |
| | Packaging | Foot mat, boxes, weight, blade, trolley, lift truck, etc. | 66 |
| | Shared accommodation | Remote control, fridge, kettle, light switch, cabinet knob, etc. | 114 |
| | Private accommodation | Remote control, door handle, washing basin, wall, etc. | 56 |
| | Vehicles | Car, forklift, tractor | 34 |
| | Office restricted access | Personal electronic devices | 8 |

#### 2.2.1. Sampling of Clothing, Protective Items and Other Fabrics

For sampling, seven employees were supplied with white cotton T-shirts, which were washed only once after purchase. At the end of the working day, these plant sap-stained shirts were collected and packed separately. Disposable latex gloves worn by the employees were also collected and investigated. Shoe covers worn for a few hours as well as the bedding of employees were also examined for ToBRFV contamination. Factory-new, unused disposable gloves and shoe covers and a T-shirt served as negative controls. Samples were packed separately in sealable plastic bags and stored at 6 °C until being tested in bioassays.

Standardized subsamples of 3 × 3 cm were cut out from each object immediately prior to inoculation in the bioassay. This procedure was standardized to consider differently contaminated areas. For example, in the case of T-shirts, the sleeves, hip and abdominal areas, back, neck, and chest were considered, and in the case of gloves, the back and palm sides of the fingers, as well as the back of the hand, were tested (Figure 1).

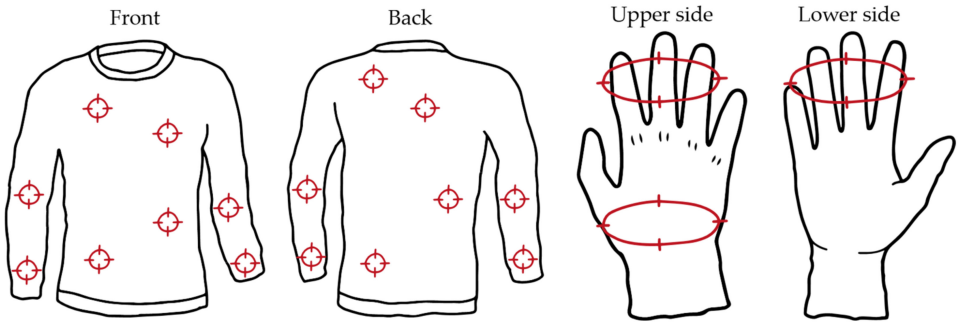


**Figure 1.** Position of subsamples taken from T-shirts and gloves. A total of 16 samples from each T-shirt and three samples from each glove were processed.

#### 2.2.2. Sampling of Swab Samples

To assess the risk of infectious ToBRFV particles being carried around the farm, swab samples were taken. To do this, viscose swabs in a tube (LxØ 108 × 16 mm) were used (Sarstedt AG & Co. KG, Nürmbrecht, Germany). Immediately before swabbing, the stick was moistened with buffer (0.1 M $Na_3PO_4$, 0.2% $Na_2SO_3$, pH 7.0) and then wiped over the sampling area within a standardized area of 10 $cm^2$. For this purpose, 2 × 5 cm PETG templates (Figure 2A) produced by a 3D printer were used. The surfaces of the templates were disinfected with pursept A (wolk AG, Wuppertal, Germany) after each contact with a

sampling area to avoid any cross-contamination. The sticks were sealed in the tube and stored until tested in a bioassay at 6 °C.

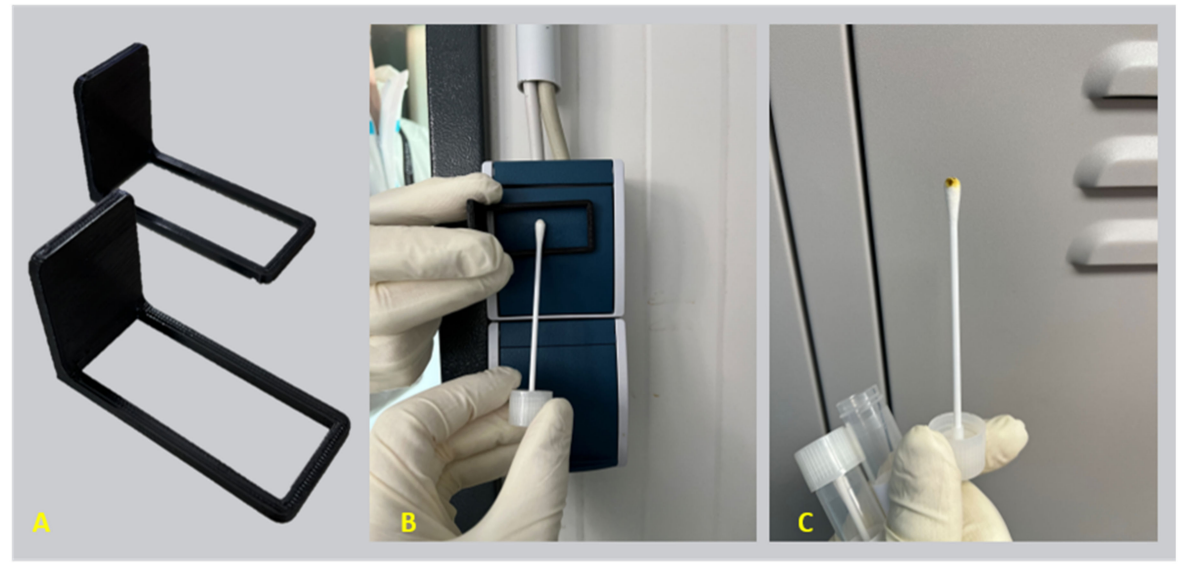


**Figure 2.** Procedure for sampling with wipe samples in a ToBRFV-infested tomato farm. (**A**) Templates of 2 × 5 cm size made in a 3D printer. (**B**) Sampling of a suspected contaminated light switch with a moistened viscose swab on a defined surface. (**C**) Viscose swab after sampling.

A total of 356 samples were taken from the production area (greenhouses), packaging hall, accommodation, and other company properties such as an office and company vehicles (Table 1). Since only a fraction of the operating surfaces can be sampled with the swab samples, switches, handles, and knobs, which are frequently touched by potentially contaminated hands, were sampled.

### *2.3. Detection of ToBRFV by Different Detection Methods*

#### 2.3.1. Detection of ToBRFV by RT-PCR

Initially, symptomatic tomato fruits were tested for the presence of ToBRFV by RT-PCR (reverse transcription polymerase chain reaction) using the ToBRFV-1482-s/ToBRFV-1677-as primer pair, as previously described [44], to ensure that ToBRFV was still present in the tomato crop at the time of sampling. Total RNAs from tomato fruits were extracted using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Catalog No. STRN50, Hilden, Germany) and integrated with DNase I using an On-Column DNase I Digest Set (Catalog No. DNASE10 and DNASE70, Hilden, Germany) following the manufacturer's instructions.

#### 2.3.2. Detection of ToBRFV and Proof of Infectiosity by Bioassay

For the detection and confirmation of the infectivity of ToBRFV in the bioassay, *Nicotiana tabacum* L. cv. Xanthi NN was used as an indicator plant. Test plants were grown in a greenhouse under controlled conditions (20 °C/16 °C day/night and 16 h/8 h light/dark) in pots (Ø 9 cm) filled with bedding substrate (Klasmann-Deilmann GmbH, Geeste, Germany). Cultivation and crop protection were carried out as previously described [35]. For the bioassays, *Nicotiana tabacum* cv. Xanthi NN was mechanically inoculated with the samples and produced necrotic local lesions when successfully inoculated with the virus. For this purpose, leaf halves were first injured with abrasive diatomaceous earth [CAS 61790-53-2], and for each sample, three leaf halves of an indicator plant were subsequently inoculated. Visual evaluation was performed 6–7 days after inoculation (dai). The presence and infection of ToBRFV were assumed when the first characteristic necrotic local lesions appeared on one of the three inoculated leaf halves.

2.3.3. Detection of ToBRFV by DAS-ELISA

To ensure that necrotic local lesions were indeed induced by ToBRFV and not by other plant viruses, composite samples of inoculated *N. tabacum* cv. Xanthi NN leaves were taken from sampled objects/surfaces and serologically tested in a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with the commercially available assay RT-1236 (DSMZ) according to the manufacturer's instructions [45] as described earlier [36].

### *2.4. Statistical Analysis*

The aim was to describe the situation of ToBRFV contamination on surfaces in an infested tomato farm for the first time. Therefore, the focus was mainly on descriptive statistics in tables and plots. In a few cases, a comparison of infection frequency was performed using Fisher's exact test or the $Chi^2$ test in the context of a contingency table (with alpha = 0.05) using the FREQ procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

## 3. Results

### *3.1. Detection of ToBRFV by RT-PCR*

The samples of symptomatic tomato fruits were tested ToBRFV positive by amplifying a 196 bp fragment, which was expected. The presence of ToBRFV on the entire farm was therefore assumed.

### *3.2. Frequency of ToBRFV-Contamination on Surfaces in Different Farm Locations*

The presence of dried plant sap, green to brown in color, on tested surfaces was only a limited indicator for the presence of ToBRFV contamination in the sampled tomato farm (Figures 3 and 4). We used Fisher's exact test to determine whether the ratio of ToBRFV-contaminated to non-contaminated surfaces could be inferred from a visible plant sap stain. In fact, 46 of 70 (65.7%) samples taken from the tomato production-related areas of greenhouses, packaging and office areas, and farm vehicles were contaminated with ToBRFV, although they displayed visible plant sap contamination during sampling. However, it was found that 19.8% (23 of 116) of the sampled surfaces in these areas with no visible plant sap residue were contaminated with enough virus to cause infection of the test plants.


**Figure 3.** Objects and surfaces from which swab samples were taken for possible contamination with ToBRFV. '(**A**)' = hand rail; '(**B**)' = door handle; '(**C**)' = green sap stained wall; '(**D**)' = light switch; '(**E**)' = kettle; '(**F**)' = cabinet handle; '(**G**)' = foot mat; '(**H**)' = trolley; '(**I**)' = pipe; '(**J**)' = foil; '(**K**)' = controls of lift truck; and '(**L**)' = lift truck.

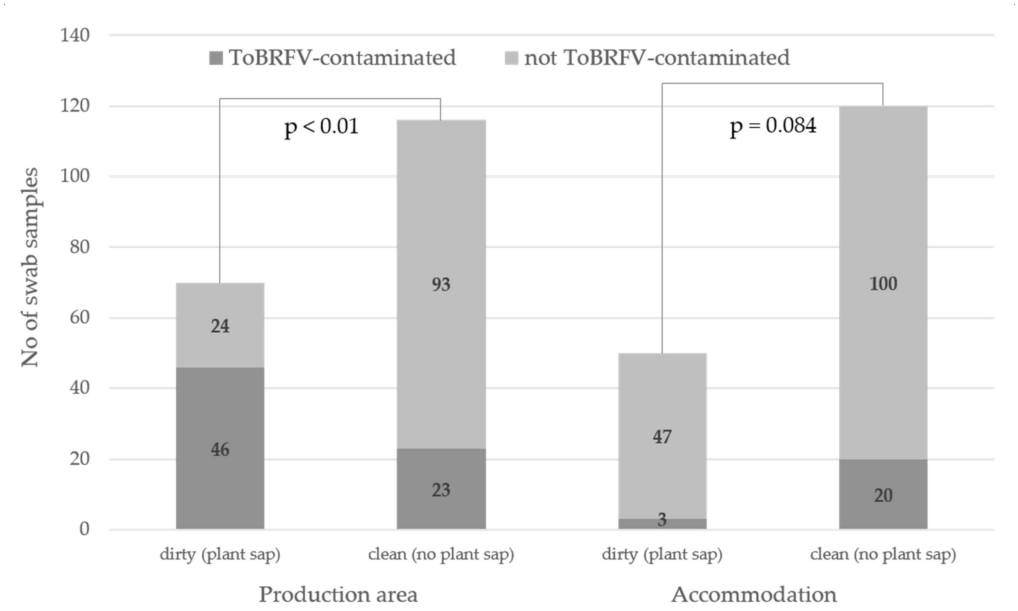


**Figure 4.** Number of ToBRFV-contaminated swab samples taken from different surfaces in the production area and accommodation dependent on visual staining with plant sap or plant debris determined by mechanical inoculation of the indicator plant *Nicotiana tabacum* cv. Xanthi NN with swab samples. 'Production area' = greenhouse, packaging hall, vehicle, and office (restricted access). 'Accommodation' = shared and private accommodation. *p* value from Fisher's exact test comparing the ratio of ToBRFV-contaminated to non-contaminated surfaces between visible dirty and clean surfaces.

Compared to the production area, a relatively lower number of ToBRFV-contaminated surfaces were found in the accommodation area. In total, 23 of the 170 samples taken here were contaminated with ToBRFV (Figure 4). However, the majority of these ToBRFV positive surfaces were not soiled with plant sap or plant residues and were therefore visually "clean" (20 of 120). Only 3 of the 50 samples simultaneously showed visible contamination in combination with ToBRFV infection of the test plants.

For this ToBRFV-infested farm, we can conclude that the surfaces in the production area stained with plant sap were more frequently contaminated with infectious ToBRFV ($p < 0.001$), while no significant difference between "dirty" and "clean" surfaces was found in the accommodation area ($p = 0.084$).

As expected, the greenhouses in which tomatoes were cultivated were most heavily contaminated with the tobamovirus. Almost half (48.7%) of all taken samples were highly contaminated with ToBRFV, so inoculation with the sample swabs led to infection of the test plants (Table 2). The high number of positive ToBRFV samples from machines, pipes, or even plastic foil is clear, since these surfaces or objects were in direct contact with potentially infected tomato plants. However, the frequency of contaminated samples from the greenhouse tracks, as well as from the foot mat, demonstrates that there has been a carry-over of the virus, e.g., through shoe soles. The packaging hall was contaminated with the virus to almost the same extent (40.9%). However, a clear separation between the greenhouses and the packaging hall cannot be drawn, since some items from the packaging hall were also used in the greenhouse, e.g., pruning shears.

Beyond these locations related to tomato production, ToBRFV was also found in the accommodations used by the employees. Especially the shared areas were highly contaminated with ToBRFV (18.4% positive), whereas the private rooms were less contaminated (3.6% positive). In the shared rooms, various objects, such as handles of cabinets, doors, and large electrical devices, as well as switches of kettles or light switches, were contaminated with ToBRFV. Interestingly, samples taken mostly from walls heavily soiled with green plant sap did not show any infectious virus contamination (Figure 3c). In the private

accommodation consisting of six double rooms, which were standardized sampled there was hardly any contamination found at all. Only two remote controls for the televisions were contaminated with ToBRFV at the on/off button, which is used more frequently. Other parts of the farm sampled, such as vehicles or the office, which could only be entered by the plant manager, were barely contaminated or not contaminated at all (Table 2).

**Table 2.** Frequency of ToBRFV-contaminated samples from different company locations determined by mechanical inoculation of the indicator plant *Nicotiana tabacum* cv. Xanthi NN with the swab sample.

| Location | Sampled Object | Total | ToBRFV-Contaminated Samples | |
|---|---|---|---|---|
| | | | [n] | [%] |
| Greenhouses | Road surface | 18 | 10 | 55.6 |
| | Foil | 12 | 8 | 66.7 |
| | Pipes | 6 | 3 | 50.0 |
| | Tracks | 4 | 3 | 75.0 |
| | Machines | 14 | 8 | 57.1 |
| | Foot mat | 4 | 4 | 100 |
| | Construction | 6 | 0 | 0 |
| | Hanging channel | 6 | 1 | 16.7 |
| | Other | 8 | 1 | 12.5 |
| | ∑ | 78 | 38 | 48.7 |
| Packaging | Foot mat | 20 | 7 | 35.0 |
| | Transport boxes | 10 | 2 | 20.0 |
| | Weight | 8 | 3 | 37.5 |
| | Pruning shears | 6 | 6 | 100 |
| | Personal electronic devices | 4 | 0 | 0 |
| | Machines | 18 | 10 | 55.6 |
| | ∑ | 66 | 28 | 40.9 |
| Shared accommodation | Floor | 18 | 4 | 22.2 |
| | Wall | 16 | 0 | 0 |
| | Handle | 30 | 7 | 23.3 |
| | Switches | 24 | 3 | 12.5 |
| | Seat | 10 | 1 | 10.0 |
| | Sink | 6 | 3 | 50.0 |
| | Fabrics | 4 | 0 | 0 |
| | Other | 6 | 3 | 50.0 |
| | ∑ | 114 | 21 | 18.4 |
| Private accommodation | Switches | 12 | 0 | 0 |
| | Handle | 18 | 0 | 0 |
| | Remote control | 6 | 2 | 33.3 |
| | Wall | 8 | 0 | 0 |
| | Sink | 12 | 0 | 0 |
| | ∑ | 56 | 2 | 3.6 |
| Vehicle | Car | 20 | 1 | 5.0 |
| | Forklift | 10 | 2 | 20.0 |
| | Tractor | 4 | 0 | 0 |
| | ∑ | 34 | 3 | 8.8 |
| Office restricted access | Personal electronic devices | 8 | 0 | 0 |
| Whole farm | All | 356 | 92 | 25.8 |

### *3.3. Effect of Surface Material on the Frequency of ToBRFV Contamination*

In addition to visual contaminations such as plant sap and spatial proximity to the origin of ToBRFV contamination, the infected tomato crop, the impact of different materials on carry-over, and the persistence of ToBRFV on the farm were also investigated.

In this study, no significant differences were observed between plastic, steel, and other materials with regard to ToBRFV contamination in each location (Table 3). In total, 30.2% of the sampled plastic surfaces, 30.0% of the sampled steel surfaces, and 25.0% of other materials were contaminated with infectious ToBRFV in the greenhouse, packaging hall, and accommodation.

**Table 3.** Proportion of ToBRFV-contaminated samples with regard to different surface materials determined by mechanical inoculation of the indicator plant *Nicotiana tabacum* cv. Xanthi NN with swab samples. *p* value from Fisher's exact test comparing the ratio of ToBRFV-contaminated to non-contaminated surfaces of different materials.

| Location | Surface Material | ToBRFV-Contaminated Samples | | |
|---|---|---|---|---|
| | | Total | [n] | [%] |
| Greenhouse | Plastic | 26 | 15 | 57.7 |
| | Stainless steel | 22 | 8 | 36,4 |
| | Others | 30 | 15 | 50.0 |
| | | | $Chi^2 = 2.20$ $p = 0.333$ | |
| Packaging | Plastic | 22 | 7 | 31.8 |
| | Stainless steel | 24 | 14 | 58,3 |
| | Others | 20 | 7 | 35.0 |
| | | | $Chi^2 = 3.95$ $p = 0.139$ | |
| Shared accommodation | Plastic | 44 | 11 | 25.0 |
| | Stainless steel | 26 | 5 | 19,2 |
| | Others | 44 | 5 | 11,4 |
| | | | $Chi^2 = 2.74$ $p = 0.255$ | |
| Private accommodation | Plastic | 24 | 2 | 8.3 |
| | Stainless steel | 18 | 0 | 0 |
| | Others | 14 | 0 | 0 |
| | | | $Chi^2 = 2.202$ $p = 0.333$ | |
| ∑ | Plastic | 116 | 35 | 30.2 |
| | Stainless steel | 90 | 27 | 30.0 |
| | Others | 108 | 27 | 25.0 |
| | | | $Chi^2 = 0.91$ $p = 0.635$ | |

### *3.4. ToBRFV Contamination on Work Clothing*

In order to understand the course of the spread of ToBRFV from infected plants to distant areas of the farm, employee clothing and protective items were tested for ToBRFV. The outer clothing was heavily contaminated with ToBRFV after only one working day. The T-shirt of each employee was successfully used to infect test plants (Figure 5). For two of the seven T-shirts worn, all 16 fabric subsamples cut out were ToBRFV positive, and on five other T-shirts, 15 of each of the 16 subsamples were contaminated with ToBRFV. The subsamples without ToBRFV came from random positions: the front and back of the T-shirt, as well as the sleeves and torso. As expected, the T-shirt, which was not worn by an employee in the greenhouse, was completely free of ToBRFV.

### *3.5. ToBRFV Contamination on Protective Items*

In addition to the T-shirts, the shoe covers and latex gloves were also heavily contaminated with ToBRFV. On the gloves soiled with dried plant sap, all 20 samples from the finger area and 8 out of 10 samples from the back of the hand were positive for ToBRFV. The sole area of the shoe covers was also heavily contaminated with ToBRFV. All ten subsamples that were cut out caused ToBRFV infection on test plants. Even the upper area of the plastic cover led to infection in three of the ten subsamples (Figure 6).

### *3.6. ToBRFV Contamination on Bed and Linen*

The sleeping areas of the employees in the private accommodations were also contaminated with ToBRFV at different intensities. ToBRFV was detected on every surface in the

sleeping area tested. In particular, the pillows were frequently contaminated (10 out of 42 ToBRFV-positive samples), as was the mattress cover, with two out of 10 samples testing positive. The plant virus was even detected in parts of the foam mattress (Figure 7).

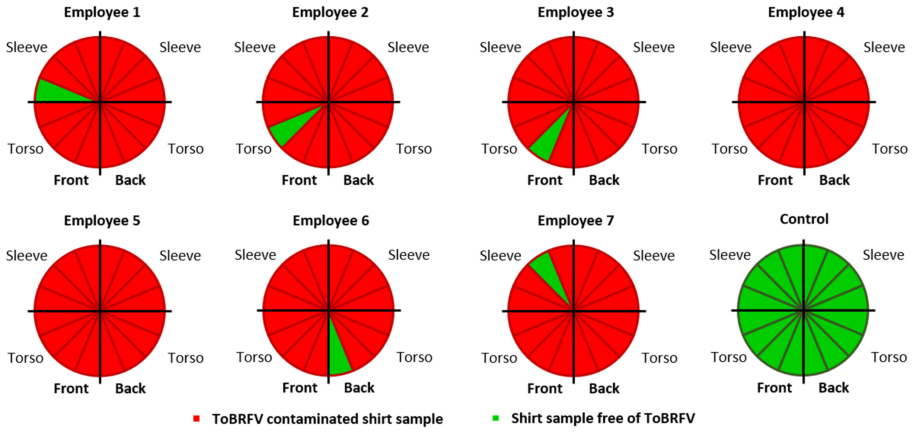


**Figure 5.** Proportion of 16 ToBRFV-contaminated and non-contaminated subsamples of each T-shirt worn by an employee for one day in a ToBRFV-infested greenhouse determined by mechanical inoculation of the indicator plant *Nicotiana tabacum* cv. Xanthi NN with fabric subsamples.

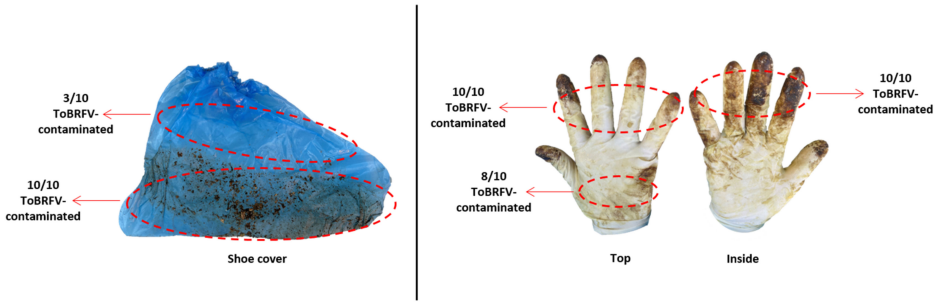


**Figure 6.** Proportion of ToBRFV-contaminated samples taken from different protective items worn for one day in a ToBRFV-infested greenhouse determined by mechanical inoculation of the indicator plant *Nicotiana tabacum* cv. Xanthi NN.

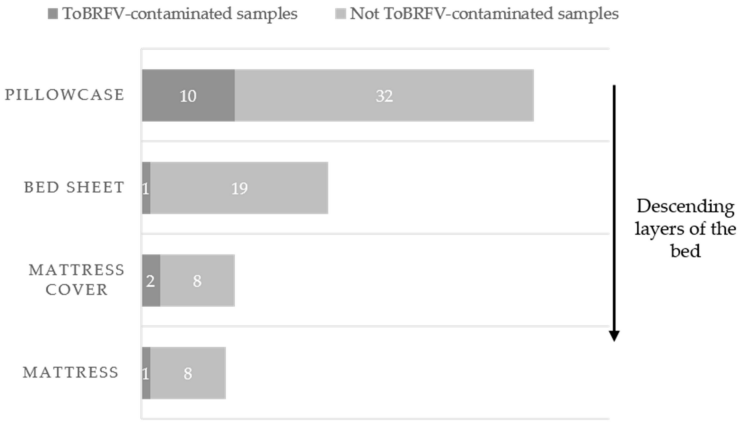


**Figure 7.** Proportion of ToBRFV-contaminated samples of different items from beds of employees in a ToBRFV-infested farm determined by mechanical inoculation of the indicator plant *Nicotiana tabacum* cv. Xanthi NN.

## 4. Discussion

Picking up a non-visible (plant) pathogen in sufficient quantity to cause infection of a healthy test plant [46] using a 10 $cm^2$ swab sample seems highly unlikely. Nevertheless, almost 26% of all randomly taken swab samples were contaminated with infectious ToBRFV

to such an extent that they were able to cause such an infection. Further samples taken from employees' clothing and protective items were highly contaminated with the plant virus. These findings are based on the data analysis of a single affected farm, and although they are not automatically applicable to other tomato-producing farms, they illustrate the tremendous risk of carryover of the persistent tomato brown rugose fruit virus on various surfaces.

In recent outbreaks of ToBRFV, the source where the virus entered the farm and caused the initial infection is often unknown, including in this outbreak. For instance, the virus may be introduced through infected planting material or fruits [40,41,47], contaminated soil [34], and especially through the changing staff, which may come from other tomato farms and introduce ToBRFV unknowingly attached to their private clothing [35,36]. Once introduced into the greenhouse, easy mechanical transmission leads to an almost complete infection of the host plant crop within a very short period of time [48–50]. Virus transmission mainly occurs through wounding of the plant, e.g., through pruning or harvesting activities. The viruses are then distributed systemically throughout the plant in the phloem system [51] and can then be further transmitted with the next contact, leading to epidemic spread in the greenhouse.

Based on this and the fact that plants cannot be cured after virus infection, as well as the difficulties of complete greenhouse disinfection in regard to tobamovirus contamination [42,52], prevention of virus introduction, and the interruption of transmission pathways are of utmost importance. Should an outbreak have already occurred, a suitable hygiene concept is crucial to maintaining fruit production as long as possible to mitigate economic losses. This was not achieved on the farm investigated in this study.

Several studies on the epidemiology of various tobamoviruses have already been published, explaining how quickly entire plant crops can be infected by phytopathogenic viruses, starting from individual or a few infected plants [49,50]. In contrast to these studies, the purpose of our sampling is to show where and with what frequency virus contamination occurs in order to conclude where cleaning and disinfection measures need to be implemented beyond the normal level.

As expected, the source of ToBRFV infection—the greenhouse filled with tomato plants—was most widely contaminated with ToBRFV. The close proximity to the neighboring building—the packaging hall—and the fact that there was no hygiene gate or assignment of employees to designated working areas is reflected in the equally high contamination of the packaging hall. Following a work shift, the workers would go to the separately located shared accommodations. This is the location with the third-highest chance of finding ToBRFV on contaminated surfaces or items. The private accommodation, which can only be entered through the shared accommodation, was not so strongly contaminated, but ToBRFV was still found in individual rooms.

The findings illustrate not only that spatial distance has an impact on the rate of ToBRFV spread, but that individuals or just one individual can be responsible for spreading ToBRFV into shared spaces, even though other workers implemented all hygiene measures. The likelihood of finding infectious ToBRFV attached to an object or a surface is highly variable and is not directly transferable to other farms. However, the different frequencies of contamination between the locations indicate a trend from the tomato production hotspot weakening toward the accommodations. The driver of this trend is inevitably human activity. This is demonstrated by the large number of ToBRFV positive samples on clothing (95.5%), gloves (93.3%), and shoes (65%) that were worn for a single working day. These findings on the spread of ToBRFV contamination should also be implemented in the official sanitation measures based on (EU) 2020/1191, last amended by (EU) 2021/1809, in such a way that specific hygiene measures are not only applied to personnel, production, tools and machinery, packaging and transport, but also explicitly include other operational areas such as accommodation in order to prevent new epidemics.

The easy mechanical transmission of tobamoviruses in crops through hands, clothing, or shoes has already been demonstrated [35,36,52,53]. These studies are extended with

the present findings on ToBRFV contamination on inanimate surfaces of an affected farm. Although characteristics such as the high stability of tobamoviruses have been known for a long time, the disinfection of surfaces from stable tobamoviruses is still challenging.

To sanitize an affected farm, all surfaces potentially contaminated with ToBRFV or other tobamoviruses must first be cleaned and then disinfected. Initially, a dry-cleaning step of, e.g., walkways or ground covers with brushes, brooms, or shovels is necessary to remove the gross soiling. This is followed by wet-cleaning with a detergent. Warm water should be used for the cleaning solution to increase the effectiveness of the cleaning action. This procedure can be used to remove organic matter from surfaces to achieve the highest efficacy with the lowest dosage of disinfectant for sufficient virus inactivation [42]. Once cleaning is finished, the cleaned surfaces should be rinsed to remove the organic substances that could interfere with the disinfectant and prevent possible inhibitory effects of the cleaning agents on the disinfectants. Only then, the pre-washed surfaces should be treated with a disinfectant. In previous studies, the only disinfectant approved for inactivation of tobamoviruses in the EU, MENNO Florades, has proven to be a very effective disinfectant with a 4–6 log reduction of viral load on greenhouse surfaces [44]. Foam (0.4 L/m$^2$) was chosen for application as it has advantages over spray application, particularly on vertical surfaces, since foam allows longer contact times and a higher amount of active ingredients per area, thus increasing the efficacy of a disinfectant. Another disinfectant with high efficacy against tobamoviruses is sodium hypochlorite [30,54,55]. However, it should also be noted that it has harmful corrosive effects on greenhouse materials. In contrast, alcohol-based disinfectants, often used in healthcare facilities, are ineffective against tobamoviruses [56].

During the cultivation season, effective hygiene measures can already be taken to limit the spread and surface contamination of ToBRFV. An infested farm should separate sections of the greenhouse area from the people working in it and set up hygiene gates. Furthermore, cultivation activities should be carried out from non-infected rows towards infected ones. Cutting tools should be dipped in a cleaning product or disinfectant after individual plants or, at the latest, after one row. Short contact times of just a few seconds hamper the disinfection of contaminated shears [57]. Therefore, it seems useful to carry several cutting tools in order to achieve prolonged contact times of several minutes for cutting tools and disinfectants. Potentially contaminated work clothing should be cleaned and then disinfected after each working day. The detergents FADEX H+, MENNO-Hortiseptclean Plus, and the disinfectant MENNO Florades cleaned ToBRFV from contaminated clothing after >10 min. [35]. Likewise, foot mats filled with a disinfectant should be placed at each entrance or exit to prevent the spreading of viruses via the soles of shoes or tires. To clean the shoes, it is required that the viruses be first removed from the sole by brushes or mechanical treading in order to remain on or in the mat. The inactivation of ToBRFV within the mat was confirmed for MENNO Florades [36]. Hands should be protected with disposable gloves, which should be changed continuously or cleaned with a detergent.

No significant differences were found between different types of sampled surface materials, such as steel or plastic, with regard to the presence of ToBRFV contamination. It seemed rather that the spatial proximity of an object to the greenhouse, as well as the frequency with which the surface was touched, was more crucial than the type of material. Coutts and colleagues found that the potyvirus Zucchini yellow mosaic virus (ZYMV) exhibited varying stability on different materials. Accordingly, ZYMV remained infectious on plastic for a longer time compared to metal [58]. However, it should be noted that studies on the (long-term) survival of plant viruses on inanimate surfaces are scarce. Extensive research on various factors affecting virus survival on surfaces has been undertaken in the field of human pathogens [59]. Multiple physical aspects that affect surface properties and viral persistence, such as porosity, absorption, and surface hydrophobicity, have already been studied in this context [60]. To address the issue of controlling tobamoviruses in daily practice, it seems particularly promising to use metal coatings with virucidal activity, such as copper or silver, or suitable polymers for handles and switches in greenhouses [60].

However, these effects, which have been confirmed for human pathogens, must first be tested for phytopathogenic viruses that are difficult to inactivate.

The fact that ToBRFV contamination could even be detected in the sleeping areas poses a particular risk since, in contrast to the greenhouses, a mattress might not be cleaned and disinfected after each season, and thus infectious ToBRFV particles might be re-introduced from the private accommodation into the new crop in the following year. It is obvious that the farm in this study did not succeed in separating the production area from the private area. It must be expected that employees will carry ToBRFV via virus-contaminated private clothing to other greenhouses and employers unless personnel-related hygiene measures are implemented there. The relatively high frequency of contamination of the pillowcases suggests another possible route of transmission and dissemination that was not investigated in this study, namely contaminated hair. Contaminated (human) hair as a route of transmission has not yet been evaluated in previous studies. Whether household shampoos lead to a satisfying cleaning of hair under phytosanitary aspects cannot currently be answered. Alternatively, hair nets could also represent a possible protection for hair. It has been demonstrated that pathogens may spread throughout a facility if hygiene plans are not properly implemented. This finding was illustrated for ToBRFV but also applies to other stable fungal, bacterial, or viral pathogens. The only way to avoid dissemination is to implement mandatory, effective hygiene measures, such as careful cleaning and disinfection.

Another aspect that will be of major relevance for upcoming sampling studies is the quantification of virus load on surfaces, i.e., how many infectious virus particles have accumulated on different surfaces. The availability of these data could be an indicator to adapt the level of intensity of cleaning and disinfection measures. The quantification of infectious ToBRFV concentrations on different surfaces has been established [44,46]. As reported, depending on the number of replicates, at least $8.37 \times 10^{-6}$–$321 \times 10^{-6}$ mg/mL of ToBRFV particles are required for the development of a local lesion on all inoculated half-leaf units of the local lesion host [46]. Thus, in the wipe samples of a 10 $cm^2$ surface that tested positive for ToBRFV in the bioassay, at least this amount of virus particles in the range mentioned must have been present. In reality, the virus contamination present will be much higher, as a significant amount of virus particles probably remained in the viscose swab after inoculation. Other swab samples, e.g., from contaminated scissors and knives, had considerably more than one lesion per test plant and therefore had a higher virus concentration [44,46,61].

## 5. Conclusions

Due to its physical properties, high thermal inactivation point, and longevity in vitro, the infectious tomato brown rugose fruit virus is able to adhere and persist on various surfaces and subsequently be picked up by humans and infect entire greenhouse crops due to its easy mechanical transmissibility. ToBRFV was detected in almost every area of the affected farm, including the accommodation areas. The spread was presumably caused by humans. These findings underline the importance of coordinated sanitation measures that include all potential transmission routes and, if possible, the strict separation of the production area from other operational areas such as accommodation. The only way to achieve this is through the use of technical equipment in the form of hygienic gates in combination with approved, effective cleaning and disinfection products, as well as by sensitizing and training employees.

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