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Decontamination of *Tomato Brown Rugose Fruit Virus*-Contaminated Shoe Soles under Practical Conditions

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Abstract: Due to its global spread, easy mechanical transmissibility inside greenhouses, and detrimental effects on marketability, *Tomato brown rugose fruit virus* (ToBRFV) is considered to be one of the biggest threats to tomato production. Regarding such crop epidemics, it is essential to identify all conceivable transmission routes and to interrupt them with effective decontamination strategies. We analyzed the potential efficacy of reliable shoe sole cleaning in combination with a disinfection measure. For this purpose, first, a suspension test was undertaken that involved applying different disinfectants to an infected plant homogenate. This was followed by a simulated carrier test. Finally, shoe-sole decontamination was tested under practical conditions. The extent of decontamination was determined by bioassays of the infectivity of the initial load remaining after treatment. Thereby, necrotic local lesions on the susceptible indicator plant *Nicotiana tabacum* cv. Xanthi NN were counted. Recommendations for practical applications, based on suspension or simulated carrier tests, are limited in their applicability, since very short contact times between ToBRFV and disinfectants reduce efficacy. Under practical conditions, the approved disinfectant MENNO Florades was able to achieve complete inactivation of the virus in the disinfection mat following mechanical depletion from the shoe soles.

Keywords: hygiene; disinfectant; disinfection mat; employees; scraping off

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1. Introduction

Tomato brown rugose fruit virus (ToBRFV), of the genus Tobamovirus, causes severe economic damage in tomato (*Solanum lycopersicum* L.) crops [1], as a result of yield reduction and discoloration and strong rugose fruit symptoms, which preclude marketing [2–4]. Since its first outbreak in Israel in 2014 [2] and Jordan in 2015 [3], ToBRFV has already been detected in many major tomato-growing countries in the northern hemisphere, such as USA [5], China [6], Mexico [7], Spain [8], Italy [9], Turkey [10] and Germany [4]. Due to a lack of curative plant protection treatments against viruses, preventive hygiene and disinfection measures are crucial to fulfill phytosanitary and economic demands. Otherwise, all host plants would eventually become infected [3,11]. Experiences from previous ToBRFV outbreaks confirm that transmission is caused by not only cutting tools but also other transmission paths, in particular through humans. Therefore, cleaning tests have already been carried out on clothing [12], which is extended by investigations of the disinfection of shoe soles in this study.

To date, investigations of the decontamination of shoe soles mainly focus on healthcare/hospital facilities [13,14], tourism [15,16], and veterinary/livestock [17–19], while in many studies, no effective decontamination strategies against various pathogens could be demonstrated [20]. In the field of stable plant viruses, such studies are not available, although numerous (official) consultants, insurances (pers. communication), and even

authors recommend the use of such disinfection mats for the inactivation of these viruses on shoe soles or tires [21–23].

Throughout the years, various tobamoviruses have presented growers with major challenges, resulting in particular from their physical properties, the very high thermal inactivation point, and longevity in-vitro and on fabrics or surfaces [24], as well as through multiple transmission pathways, such as seeds [25,26], soil [27–29], bumblebees [30], and simple mechanical transmission [12,31], resulting in an exponential spread of the viruses through, e.g., cultivation measures in greenhouses [11,32]. Moreover, inactivation through cleaning and disinfection measures is a somewhat challenging approach to integrate into the daily production routine. However, since the *Tm*-2^2 resistance gene, which used to be protective against tobamoviruses, has been broken by ToBRFV [2], evaluations of disinfection methods and related products have become more important than ever.

Unlike other tobamoviruses, such as *Tomato mosaic virus*, controlling ToBRFV is additionally aggravated by the fact that it has been regulated in many tomato-producing countries and regions as a quarantine pest or through emergency measures [33]. However, with the amendment of Commission Implementing Regulation (EU) 2021/1809 on 13 October 2021, tomato production may continue in greenhouse areas intended for the production of specified fruits, provided that, at least at the end of the growing season, the removal and destruction of all specified plants from the production area is carried out and specific hygiene measures are applied to employees, buildings in production areas, tools and machinery, materials, packaging, and means of transport for the fruits in order to prevent the spread of the specified pest ToBRFV to subsequent crops or to other production areas [34]. At present, sanitation seems to be the method of choice here.

In the last decade, several studies have shown the efficacy of using disinfectants to inactivate plant viruses [35–37]. However, these studies only investigated the inactivation of virus particles in suspension (e.g., infected plant sap). In contrast to the methodology of these studies, the products must be applied to potentially contaminated unlived surfaces in greenhouses, transport, and processing facilities. In order to address practical conditions and to be able to provide recommendations for controlling the virus, the following aspects will be examined: Which product enables sufficient effectiveness for the inactivation of ToBRFV on natural rubber surfaces? Which experimental design is suitable for evaluating the effectiveness of such a product under real-world conditions?

International requirements for standardized disinfectant assays in terms of consistency and reliable indications require the evaluation of several test systems, such as suspension, surface/germ carrier, and field tests [38]. In particular, the contact times required for reliable inactivation of pathogens by the applied disinfectant often differ considerably between the suspension and carrier tests [39]. This is why, for example, a disinfectant according to the EN 14885:2020 standard of Technical Committee 216 (Chemical disinfectants and antiseptics—Application of European standards for chemical disinfectants and antiseptics) has to pass three test stages: (i) a suspension test under laboratory conditions, which cannot be used for product claiming (phase 1), (ii) carrier tests to simulate the intended use (phase 2), and (iii) field test under practical conditions (phase 3).

The aim of this study is to demonstrate a standardized methodological approach for the evaluation of disinfectants with virucidal efficacy in disinfection mats for agricultural or horticultural practice. To this end, various products are first tested for their efficacy against ToBRFV in a suspension test and subsequently in a carrier test. Finally, we used the example of the approved disinfectant MENNO Florades to test its efficacy under practical conditions.

2. Materials and Methods

2.1. Source of ToBRFV and Plant Material

The ToBRFV isolate PV-1236, which was used in this study as well as in previous experiments [12], was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and originated from the first ToBRFV

outbreak in Germany in 2018 [4]. *Nicotiana clevelandii* A. Gray served as the host plant for virus propagation. After mechanical inoculation, the tobacco plants showed systemic infection. The infected leaf material was collected and subsequently homogenized with liquid nitrogen in a mortar using a pestle. This homogenate was used for all series of experiments and stored at −20 °C until use.

To confirm the infectivity of ToBRFV in the bioassay, we used *Nicotiana tabacum* L. cv. Xanthi NN as an indicator plant. All 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 [12].

2.2. Inoculum Preparation and Mechanical Inoculation

To prepare the inoculum for Experiments 1 (suspension test) and 2 (simulation) (Table 1), infected *N. clevelandii* homogenate was ground 1:5 (*w:v*) with autoclaved tap water in an extraction bag (Bioreba AG, Reinach, Switzerland), then transferred to 50 mL centrifuge tubes (Sarstedt AG & Co. KG, Nürmbrecht, Germany), and kept on ice until use. Immediately before mechanical inoculation, abrasive diatomaceous earth non-washed [CAS 61790-53-2] was either added to the suspension (Experiment 1) or dissolved in water and pipetted onto the leaves subsequently to be inoculated with the contaminated germ carriers (Experiment 2). All the repetitions were conducted under similar conditions with respect to the physiological stage of the test plant *N. tabacum* cv. Xanthi NN (approximately the 10–11 leaf stage) and by the same individuals. Each time, equally old leaves were inoculated.

Table 1. Overview of the preparation of different inocula used in experiments 1–3 testing the efficacy of disinfectants against *Tomato brown rugose fruit virus*.

Experiment	Plant Species	Inoculum		
		Carrier Substance	Dilution	Consistency
(1) Suspension	*N. clevelandii* A. Gray	tap water	1:5 (*w:v*)	liquid
(2) Simulation		tap water	1:5 (*w:v*)	liquid
(3) Practical conditions		quartz sand	1:10 (*w:w*)	granular

The inoculum for the 3rd experiment (test under practical conditions) (Table 1) was not a virus suspension or plant homogenate but homogenized dried-very-finely (dust) ToBRFV-infected *N. clevelandii* leaf material, which was mixed with medium-grain (0.1–0.4 mm) quartz sand in a ratio of 1:10 (*w:w*). This quartz sand inoculum mixture was transferred to a 50 mL centrifuge tube, mixed with a shaker for 5 min and then transferred to a propagation crate where contamination of the germ carriers later took place. Immediately before mechanical inoculation, the abrasive diatomaceous earth non-washed was dissolved in water and pipetted onto the leaves subsequently to be inoculated with the contaminated germ carriers.

2.3. Germ Carriers

The germ carriers made of natural rubber with a size of 7 cm^2 made of natural rubber were cut out of a cargo mat with a profile depth of 2 mm (A.T.U. Art. No: NO0013, Weiden in der Oberpfalz, Germany). In the standardized process of Experiments 2 and 3, they were representative of a shoe sole.

2.4. Selection of Disinfectants and Preparation of Disinfectant Solutions

Five different substances and substance mixtures were tested for their ability to inactivate ToBRFV, some also in different concentrations (Table 2): the plant protectant MENNO Florades (MF) (MENNO Chemie Vertrieb GmbH, Norderstedt, Germany), the biocide Virkon S (VS) (Lanxess Deutschland GmbH, Köln, Germany), the universal cleaner DanKlorix (DK) (CP GABA GmbH, Hamburg, Germany), skim milk powder (SMP) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), and Floradix Lactoferin (FD) (Salus Haus Dr. med. Otto Greither Nachf. GmbH & Co. KG, Bruckmühl, Germany).

Table 2. List of products, their active ingredients, and the tested concentration of the working solution for inactivating *Tomato brown rugose fruit virus* (ToBRFV).

Product		Working Solution
Trade Name	**Active Ingredient**	**(%)**
MENNO Florades (MF)	9% (*w:v*) benzoic acid	4
Virkon S (VS)	45.3% potassium Peroxymonosulfate	1
Skim milk powder (SMP)	45–54% lactose	10 20
DanKlorix (DK)	2.8% sodium hypochlorite	12.5 25
Floradix Lactoferrin (FD)	31% (*w:w*) lactoferrin	0.5

All working solutions were freshly prepared with deionized water (pH 5) and adjusted to the required working concentration, based on the manufacturer's instructions, other studies [35,36,40], and from grower experiences.

2.5. Experimental Design

The experiments were performed as completely randomized studies under controlled conditions in a greenhouse in 2021/22 and were repeated three times. In all experiments, 3 leaf halves were inoculated on each of 8 plants per repetition of treatment, resulting in 24 individual scores for each treatment repetition. Therefore, the obtained results of this study are based on a total of 552 inoculated plants, with 1656 inoculated leaf halves, separated into 3 experiments (Table 3). In addition to the inoculated plant/leaf halves shown in Table 2, 3 plants of *N. tabacum* cv. Xanthi NN were mock-inoculated per treatment and served as a negative control, which was exclusively treated with the putative disinfectants only, and not with virus particles. Positive control was always represented by 'Control' treated with deionized water.

2.5.1. Experiment 1: Suspension Decontamination (Laboratory Conditions)

The inoculum was prepared as described in Section 2.2 and mixed in petri dishes (diameter 60 mm) with the same volume of a double-concentrated disinfectant solution or autoclaved tap water (Control). After a defined contact time of 10 min, an aliquot of 150 µL was mechanically inoculated onto three halves of the *N. tabacum* cv. Xanthi NN leaves of each plant.

2.5.2. Experiment 2: Simulation—Boot Decontamination (Laboratory Conditions)

The inoculum was placed in a petri dish (diameter 60 mm). The natural rubber carrier was dipped into the inoculum for 5 s. Then, the contaminated carrier was dabbed 10 times on a foil to simulate footsteps on a disinfection mat. Cellulose sponges were placed in plastic trays (19 × 19 cm) and soaked to the upper edge with the disinfectant solutions or deionized water. The sponge represents a disinfection mat. The germ carriers were pressed into these sponges for 30 s each and then directly inoculated onto three leaf halves of one *N. tabacum* cv. Xanthi NN plant per germ carrier.

Table 3. Factors and scope of the different experiments.

Description	Experiment 1 Laboratory Conditions Suspension	Experiment 2 Laboratory Conditions Simulation	Experiment 3 Practical Conditions 3.1 Shoe Sole	Experiment 3 Practical Conditions 3.2 Disinfection Mat
Experimental factors (non-italic style) **or** ***constant experimental conditions*** *(italic style)*				
Product	Control, MF, VS, SMP (10, 20), DK (12.5, 25)	Control, MF, VS, SMP (10), DK (12.5), FD	Control, MF	Control, MF
Tested material/ Germ carrier	*plant sap*	*natural rubber*	*natural rubber*	*dirt suspension*
Contact time	*10 min*	*30 s*	*undefined*	1d/4d
Mechanical cleaning	*no*	*no*	no, light, strong	*no*
Scope of experiments				
Treatments (no.) §	7	6	6	4
Trial repetition (no.)	3	3	3	3
Inoculated plants per repetition/treatment (no.)	8	8	8	8
Inoculated plants (total no.)	168	144	144	96
Inoculated leaf halves (total no.)	504	432	432	288

§ depending on product, application concentration, contact times, and mechanical cleaning procedures. 'Control' = deionized water with ToBRFV, 'MF' = MENNO Florades, 'VS' = Virkon S, 'SMP' = Skim milk powder, 'DK' = DanKlorix, 'FD' = Floradix; numbers in parentheses indicate working solution, if more than one is used (see Table 2).

2.5.3. Experiment 3: Boot Decontamination (Practical Conditions)

The experimental design considered the disinfection of shoe soles (a) and the disinfection inside the mat (b).

(a) Shoe sole disinfection

The quartz sand inoculum mixture was placed in a propagation crate (non-perforated 60 × 40 × 6 cm). The germ carriers were attached to disposable shoe covers, each at the heel and at the ball of the foot (Figure 1). To contaminate the germ carriers, standardized steps were carried out in the quartz sand inoculum mixture and, depending on the experimental variant, on the disinfection mat (90 × 90 cm Flexxomat PT, Royal Brinkmann Deutschland, Straelen, Germany) filled with MF 4% or deionized water. The boot was walked on the disinfection mat, with the adhering germ carriers standardized, either cursorily or circumferentially scraped off. The "light" stamp-off variant on the mat represented a single back-and-forth stamp-off, while the "strong" variant represented a three-fold back-and-forth stamp-off. The germ carriers were immediately removed from the shoe cover and directly inoculated onto three leaf halves of one *N. tabacum* cv. Xanthi NN plant per carrier. A disinfection mat filled only with deionized water was used for control investigations.

(b) Disinfection inside the mat

The purpose of this series of experiments was to determine the disinfection performance after the shoes were scraped off the mat. For this, the quartz sand inoculum mixture previously rubbed off from the contaminated germ carriers onto the disinfection mat was transferred to a centrifuge tube and mixed with 40 mL of liquid (1:8, *v:v*) from the inside of the used disinfection mat (Figure 2). This mixture was incubated in the dark, in the greenhouse. Samples taken from it after 1 and 4 days were immediately mechanically inoculated onto the test plants. The disinfection mat filled only with water again served as a control.

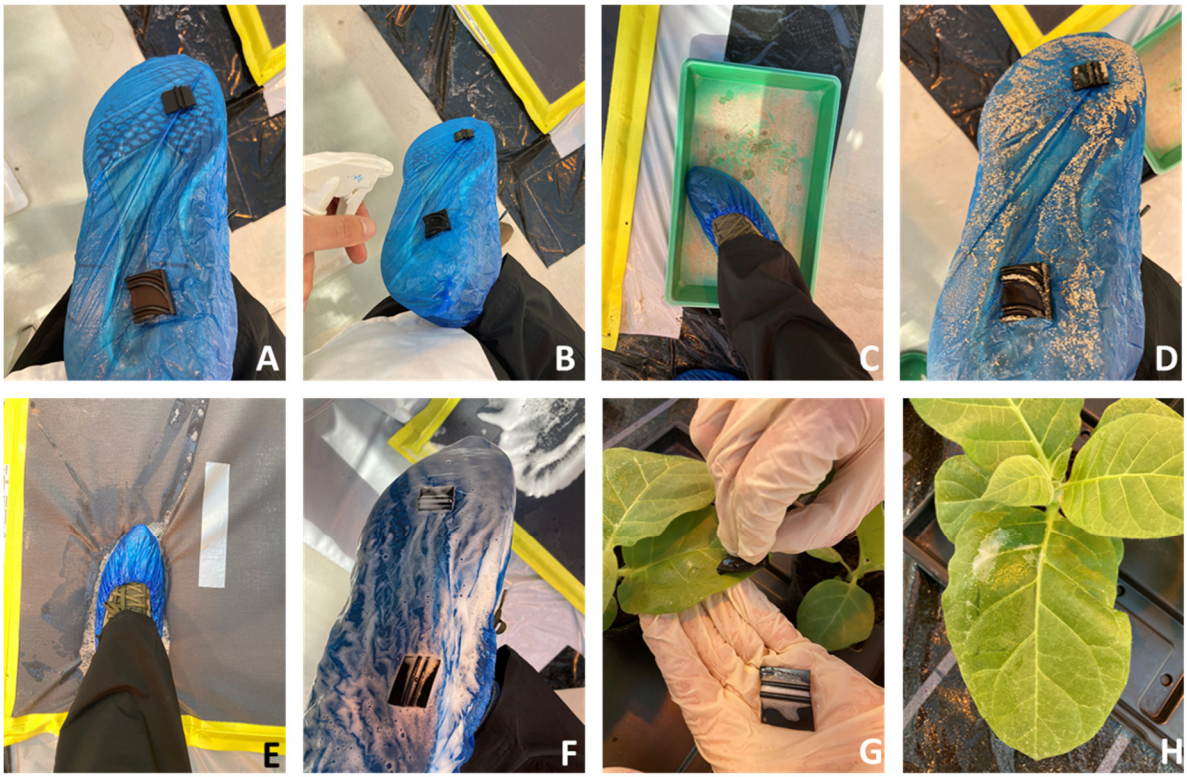

Figure 1. Illustration of boot disinfection under practical conditions. (**A**): germ carriers attached to disposable shoe covers, (**B**): germ carriers lightly moistened using a spray bottle, (**C**): step in the propagation crate with quartz sand inoculum mixture, (**D**): ToBRFV-contaminated germ carriers, (**E**): boot with the adherent germ carriers on the disinfection mat, where they are moved in a standardized manner, (**F**): germ carriers after disinfection, (**G**): inoculation of germ carrier on test plant to proof remaining infectiosity, (**H**): inoculated half leaf of a test plant.

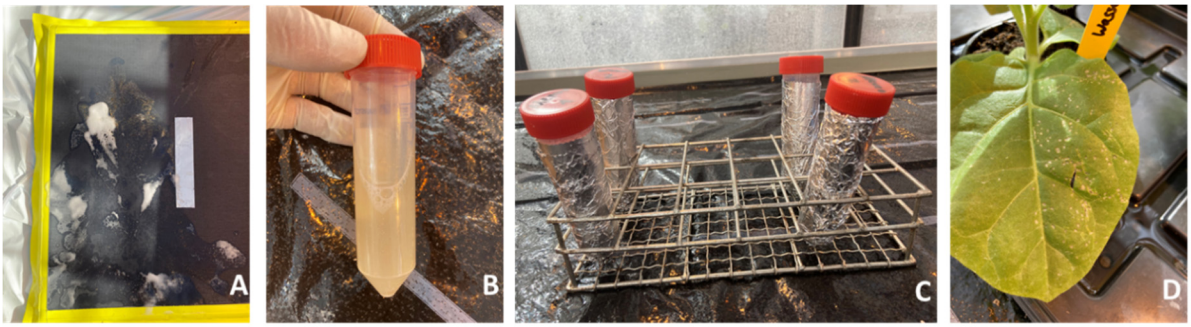

Figure 2. Illustration of disinfection performance inside the disinfection mat. (**A**): disinfection mat with layer of potentially infectious dirt derived from scraping off ToBRFV-contaminated germ carriers, (**B**): dirt mixed with liquid from the inside of the disinfection mat, (**C**): incubation of that mixture in the dark, (**D**): necrotic local lesions after the inoculation of that mixture on test plants (here water control).

2.6. Detection of ToBRFV

The number of necrotic local lesions was visually scored 6–7 days after the mechanical inoculation of leaf halves of the test plant *N. tabacum* cv. Xanthi NN. For the evaluation of the inactivation effect of disinfectants against tobamoviruses, such as ToBRFV, counting necrotic local lesions on the indicator plants is suitable, since there is a correlation between virus concentration and the number of expressed local lesions [41]. Accordingly, a relatively

high virus concentration in the inoculum induces a great number of necrotic local lesions, while a lower virus concentration leads to fewer local lesions.

Recently, this methodology was used to test different disinfectants on ToBRFV-contaminated razor blades [42] and also for a cleaning study of ToBRFV-contaminated clothing [12].

To confirm the infection with ToBRFV for all experimental approaches, composite samples of the inoculated leaf halves were taken and tested in a double antibody sandwich enzyme-linked-immunosorbent assay ELISA (DAS-ELISA) to confirm that the observed local lesions are caused by ToBRFV and do not represent any mechanical or phytotoxic damage. For DAS-ELISA, a commercially available assay was used (RT-1236, DSMZ, Braunschweig, Germany), according to the manufacturer's instructions [43]. Deviating from the protocol, 100 µL instead of 200 µL of samples and buffers were used for each well of the microtiter plate. Three replicates were tested for each sample. After 60 min substrate incubation, the optical density (OD) of the samples at 405 nm was rated. The cut-off value was defined as three times the mean value of three homogenates of different healthy (negative) samples. All samples with values above the cut-off were regarded as ToBRFV-positive.

2.7. Data Analysis

SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) was used to perform statistical analysis and for graphical illustration.

For the evaluation of efficacy, the number of lesions from leaf halves was summarized, which resulted in 24 observations per treatment (8 plants × 3 trial replications). A fixed effects model with one treatment factor (Exp. 1, 2: product) and two treatment factors (Exp. 3.1: product x mechanical cleaning, Exp. 3.2: product x time) was used to analyze the count data (0.01 was used instead of zero counts), assuming a negative binomial distribution for the residuals.

The analysis was performed within a framework of a generalized linear mixed model (GLMM; SAS procedure GLIMMIX) [44]. For all pairwise treatment comparisons, the Bonferroni correction was applied (division of alpha by the number of respective comparisons) because of the multiple-test situation. For the assessment of statistical tests, alpha = 0.05 was used.

3. Results

3.1. Suspension Decontamination under Laboratory Conditions (Experiment 1)

The chosen method of mechanical rub-inoculation reliably produced a high number of necrotic local lesions in the bioassay on the indicator plant (Table 4). The application of all tested products at the respective concentrations in a suspension test with 10 min contact time resulted in a significant reduction in the number of necrotic local lesions compared to 'Control' on *N. tabacum* cv. Xanthi NN. Thus, MENNO Florades (4%) was able to reduce the number of local lesions by 91.3% in comparison to 'Control'. For Virkon S (1%), skim milk powder (10%; 20%), and DanKlorix (25%), 98.0–99.5% fewer local lesions were detected. Differences in the number of necrotic local lesions between the two concentrations of the skim milk powder (10%; 20%) and DanKlorix (12.5%; 25%) variants were not statistically significant from each other, so that varying concentrations of active ingredient were not used in further testing of the simulated boot decontamination (Experiment 2). For the most effective treatment in this experiment, DanKlorix (12.5%), the reduction in local lesions was as high as 99.9%. Based on the results of the suspension test, following the already-described Phase 1 test for disinfectants, the second disinfection test was carried out on carriers with a simulated disinfection mat (Phase 2).

3.2. Simulated Inactivation of ToBRFV on Shoe Soles under Laboratory Conditions (Experiment 2)

Compared to 'Control' in which the natural rubber carrier was pressed in a sponge soaked with deionized water, we determined that MENNO Florades (4%) and Virkon S (1%) had no significant detectable effect on the infectivity of the stable virus particles

attached to the carrier (Figure 3). In contrast, skim milk powder (10%), DanKlorix (12.5%), and especially 0.5% Floradix Lactoferrin were able to significantly inactivate ToBRFV. The reduction in local lesions in the Floradix Lactoferin treatment compared to 'Control' was 99.3%. It should not go unmentioned that shoe-sole decontamination was carried out without previous cleaning or scrubbing of the organic material, which led to an improved effectiveness of the disinfectant in previous studies [17]. The applied methodology is rather comparable to stepping through such a disinfection mat, which is not unlikely in production routine.

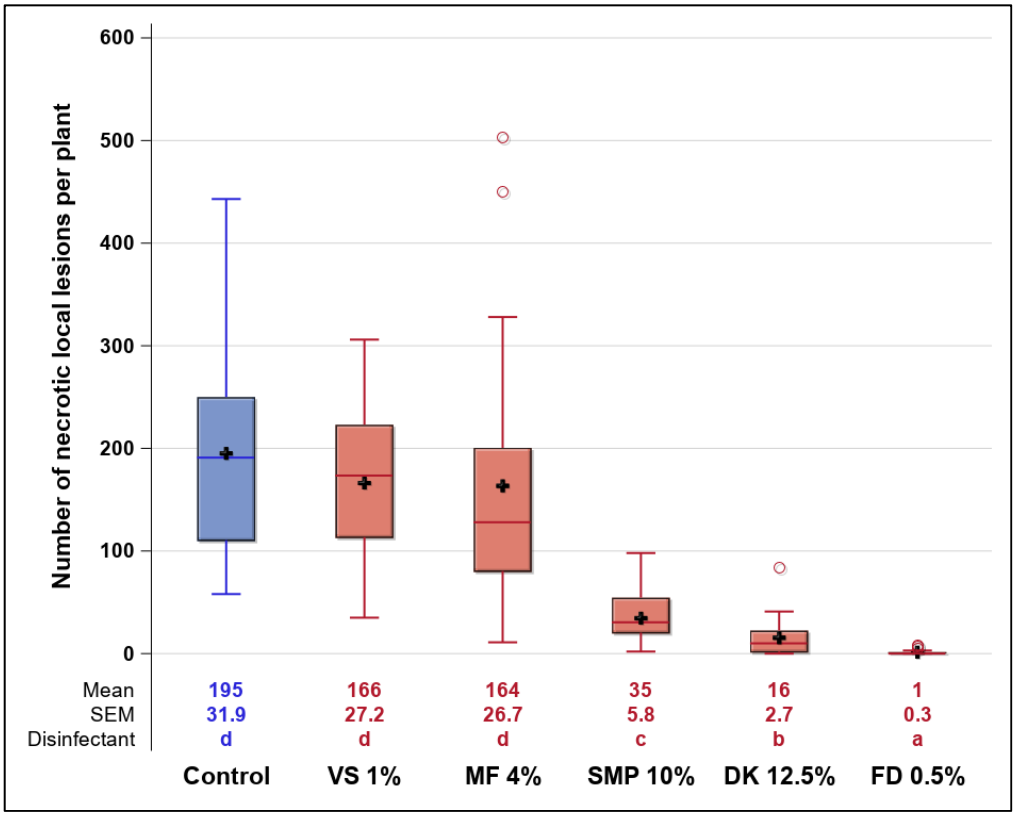

Figure 3. Evaluation of the efficiency of different disinfectants in inactivating *Tomato brown rugose fruit virus* on natural rubber at a contact time of 30 s. by counting the number of necrotic local lesions on mechanically inoculated leaf halves of *N. tabacum* cv. Xanthi NN. Observations are shown in a boxplot, cross inside = mean value. 'SEM' = Standard Error of the Mean Letters based on pairwise treatment comparisons using a generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction). n = 24. 'VS' = Virkon S, 'MF' = Menno Florades, 'SMP' = Skim milk powder, 'DK' = DanKlorix, 'FD' = Floradix.

Some modifications were made following this experiment for subsequent testing under practical conditions. First, the method of inoculum preparation was adapted to practical conditions (Section 2.5). Instead of a virus suspension, a contaminated dust/sand mixture was used as inoculum. From our point of view, the vulnerability in the second experiment was the insufficient contact time between disinfectant and ToBRFV. For this reason, in addition to the inactivation effect of the disinfectant, the mechanical removal of infectious ToBRFV particles from the contaminated shoe sole was taken into consideration. For the phase 3 test (Experiment 3), only MENNO Florades, the single plant protectant approved for this purpose in Germany, was used.

Table 4. Efficacy of selected disinfectants to inactivate ToBRFV in plant sap. Letters based on pairwise treatment comparisons using a generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction).

Treatment	No. of Inoculated Plants	No. of Inoculated Leaf Halves	Mean no. of Local Lesions	SEM	Reduction in Local Lesions Compared to 'Control'
Nc	18	54	0		100
Control	24	72	154.6 (d)	47.4	reference
MF, 4%	24	72	13.5 (c)	4.2	91.3%
VS, 1%	24	72	3.1 (b)	1.0	98.0%
SMP, 10%	24	72	2.4 (b)	0.8	98.5%
SMP, 20%	24	72	1.3 (b)	0.5	99.2%
DK, 25%	24	72	0.8 (ab)	0.3	99.5%
DK, 12.5%	24	72	0.1 (a)	0.07	99.9%

'SEM' = Standard Error of the Mean, 'Nc' = Negative control, 'Control' = Deionized water with ToBRFV-infected plant sap, 'MF' = MENNO Florades, 'VS' = Virkon S, 'SMP' = Skim milk powder, 'DK' = DanKlorix.

3.3. Shoe-Sole Decontamination under Practical Conditions (Experiment 3.1)

The usage of the disinfection mat leads to a depletion of infectious ToBRFV on the shoes. When the disinfection mat is used in combination with the disinfectant MENNO Florades, the median of 155 lesions/test plant in the control (no disinfection) drops to 25 in the case of light scraping and 15 in the case of extensive scraping of the boots (Figure 4).

The series of experiments with the disinfection mat with deionized water instead of the disinfectant MENNO Florades suggest that the depletion is physical in nature and does not result from (bio)chemical inactivation of the viruses by the disinfectant. The mean number of necrotic local lesions decreased from 156 to 15 and 5, respectively, by scraping the shoes on the mat without disinfectant. In general, the ToBRFV-contaminated germ carriers that were scraped on the disinfectant mat filled with water induced fewer lesions than those that came into contact with MENNO Florades. This is due to the inoculation behavior of the natural rubber. Compared to water, the soap-like consistency of MENNO Florades foam favors uniform rubbing of the germ carriers on the plant and penetration of the viruses into the plant. The mean values of necrotic local lesions induced on single-test plants by the respective untreated ToBRFV-contaminated germination carriers were very similar at 169 (disinfection mat with MENNO Florades) and 156 (disinfection mat with water) for this reason; the evaluation of the water and MF variants were combined.

The phenomenon—a physical depletion and no virucidal effect—was to be expected, as the contact time with the disinfectant was very short. Simply stepping on the disinfection mat led to a significant reduction. A large part of the dust could already be removed in this way. If the shoes were extensively scraped on the mat, the virus concentration on the germ carriers was reduced even further. However, complete removal of all particles is unlikely in the case of heavily contaminated dust/dirt.

3.4. Inactivation of ToBRFV Inside the Disinfection Mat under Practical Conditions (Experiment 3.2)

Disinfection inside the mat

From an epidemiological perspective, what happens to ToBRFV-contaminated dirt physically depleted from footwear is of particular importance. The infectious viruses will be in and on the mat. MENNO Florades inactivates these viruses—also in combination with organic and mineral matter (dirt)—almost completely already within one day (Figure 5). After a contact time of one day, only 2 out of 24 test plants each showed a necrotic local lesion, and after four days, none of the inoculated 72 aliquots induced a necrotic local lesion on the 24 test plants. It can therefore be assumed that the ToBRFV were completely inactivated. In contrast, water, as expected, does not lead to any notable shedding or inactivation. Although a slight but not significant reduction in necrotic local lesions

induced on the test plants was observed after four days, the dirt–water suspension was highly infectious both after one and four days and resulted in infection of all test plants after mechanical inoculation, with 166 and 107 lesions per test plant, respectively (Figure 6).

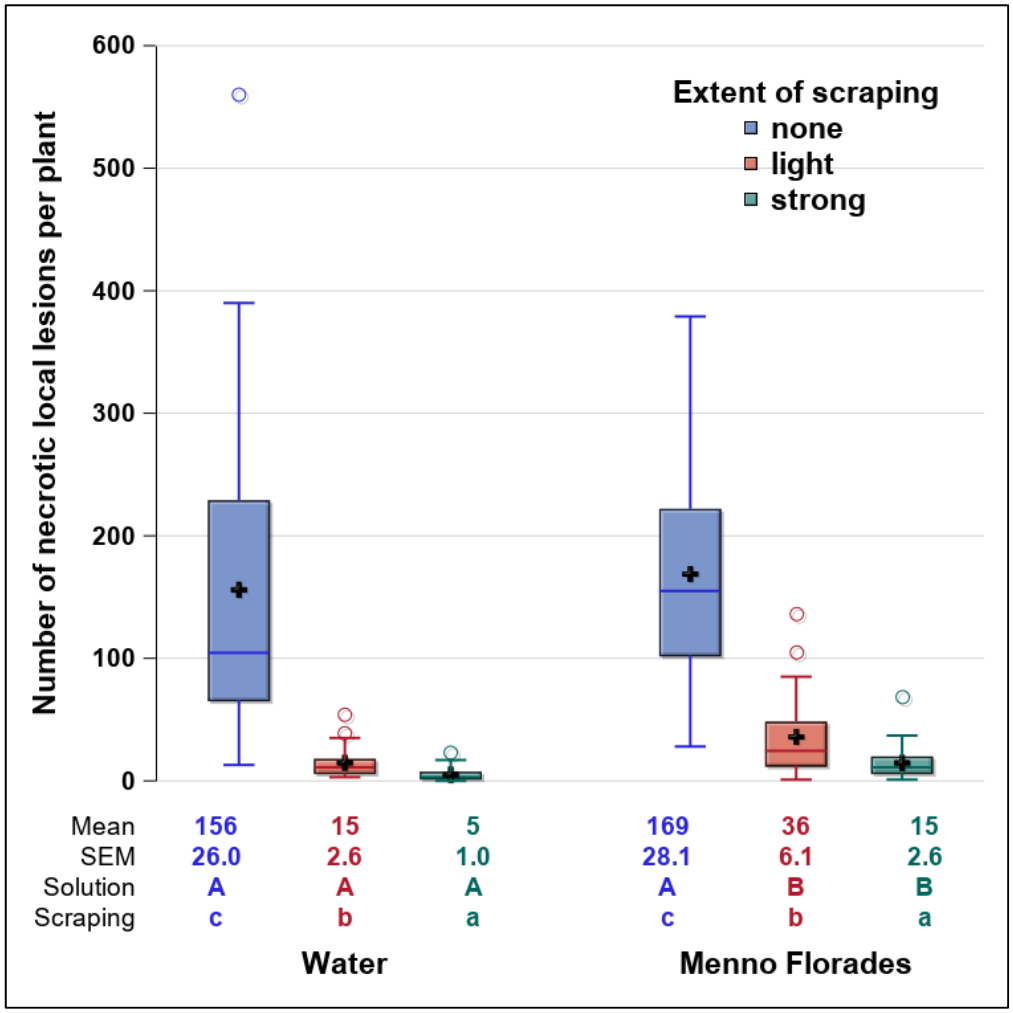

Figure 4. Efficacy of a disinfecting mat (Flexxomat PT, Royal Brinkmann, Gravenzande, The Netherlands) in combination with deionized water and Menno Florades (4%, Menno-Chemie Vertriebs mbH, Norderstedt, Germany) to *Tomato brown rugose fruit virus* (ToBRFV) contaminated natural rubber carriers depending on the extent of scraping shoes on the mat. After walking over the disinfection mat and scaping the shoe in a standardized manner, the carriers attached to it were immediately inoculated mechanically on susceptible *N. tabacum* cv. Xanthi NN to proof remaining infectiosity of the virus by counting necrotic local lesions on individual test plants. Observations are shown in a boxplot, cross inside = mean value. 'SEM' = Standard Error of the Mean. Letters (uppercase letters for the comparisons of solution within scraping levels, lowercase letters for the comparisons of scraping levels within solution) based on pairwise treatment comparisons using a generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction). n = 24.

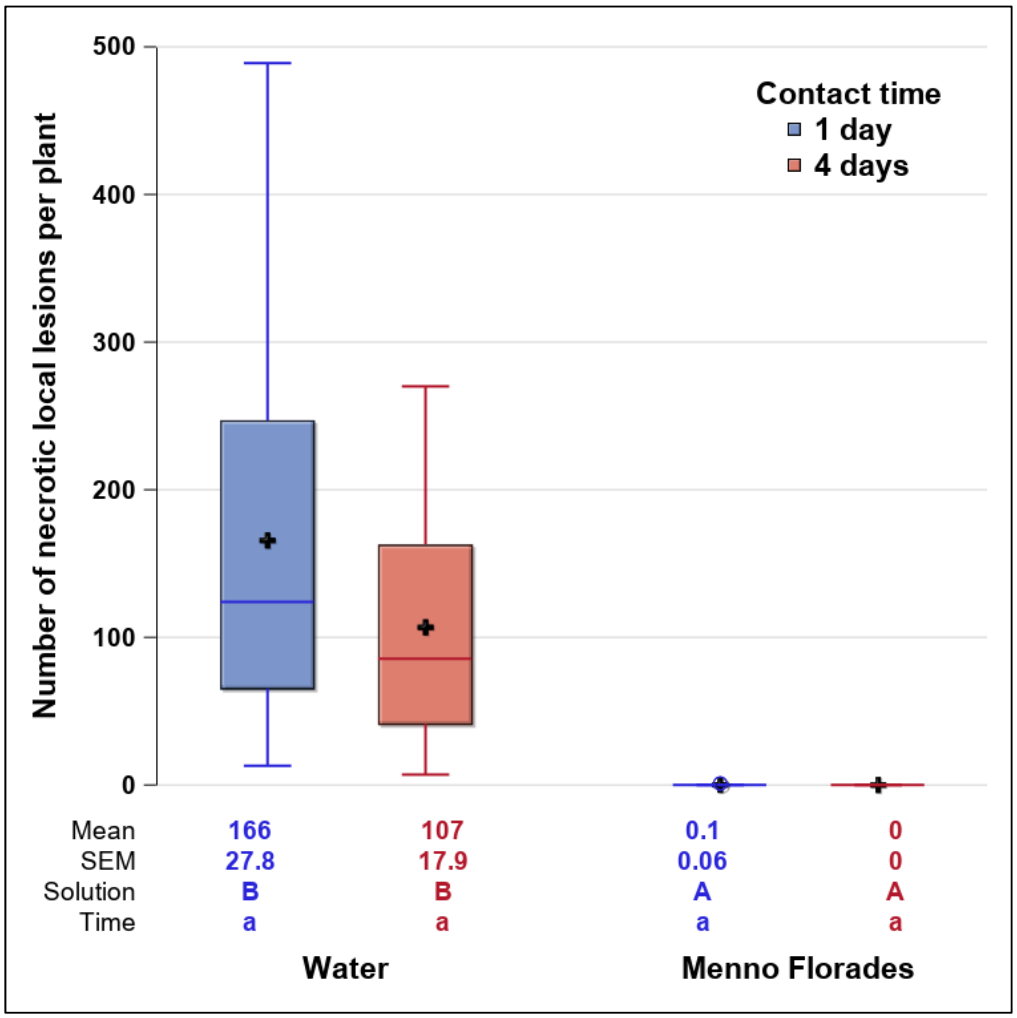

Figure 5. Efficacy of the disinfectant Menno Florades (4%, Menno-Chemie Vertriebs mbH, Norderstedt, Germany) to inactivate *Tomato brown rugose fruit virus* (ToBRFV)-contaminated dirt on a disinfecting mat (Flexxomat PT, Royal Brinkmann, Gravenzande, The Netherlands). ToBRFV-contaminated dirt was gained by natural contamination of the disinfection mat by scraping ToBRFV contaminated natural rubber (boot-disinfection). That dirt was incubated with Menno-Florades for 1 and 4 days, respectively. Dirt incubated with deionized water served as control. After the respective contact time, aliquots were inoculated mechanically on susceptible *N. tabacum* cv. Xanthi NN to determine the remaining infectiosity of the virus by counting necrotic local lesions on individual test plants. Observations are shown in a boxplot, cross inside = mean value. 'SEM' = Standard Error of the Mean. Letters (uppercase letters for the comparisons of solution within time levels, lowercase letters for the comparisons of time levels within solution) based on pairwise treatment comparisons using a generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction). n = 24.

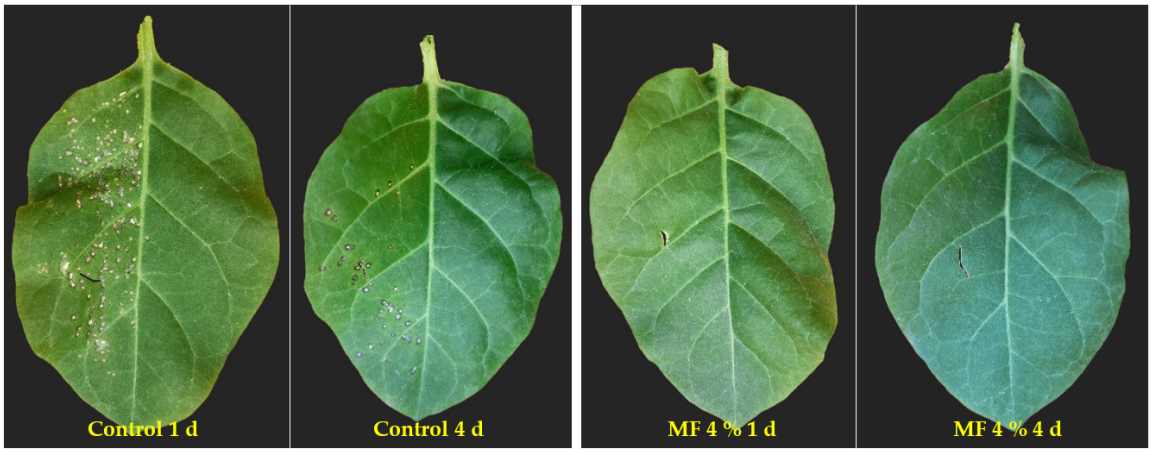

Figure 6. Comparative illustration of the efficacy of water and Menno Florades (4%) against ToBRFV within a disinfection mat for 1- and 4-days contact time, on the indicator plant *Nicotiana tabacum* cv. Xanthi NN. 'Control' = dirt + deionized water (1:8 *v:v*); 'MF' = dirt + Menno Florades (1:8 *v:v*); '1d' = one day contact time; '4d' = four days contact time.

4. Discussion

As an important part of effective hygiene, the cleaning and disinfection of shoe soles is gaining importance due to pandemics that affect human lives [45], livestock [46], or the rapid spread of *Tomato brown rugose fruit virus* in vegetable production. At the same time, these surfaces are difficult to decontaminate, since in many cases, they are loaded with organic material, which greatly weakens the effect of commonly used disinfectants, e.g., chlorine-based disinfectants [47]. Within that context, this work investigated whether ToBRFV-contaminated shoe soles can be reliably decontaminated using a disinfection mat under real-world conditions to contribute to containment of the harmful virus.

The study shows that ToBRFV-contaminated dirt adhering to shoe soles can contribute to the spread of the plant pathogenic virus and should therefore be considered, among others [12,28,42], as an important epidemiological factor. The most important finding from our experiments concerning the disinfection of ToBRFV-contaminated shoe soles is that of using the disinfectant MENNO Florades, an effective and authorized product that is already available to growers for inactivating the regulated *Tomato brown rugose fruit virus*. As shown above, the inactivation of the virus particles takes place mainly in the disinfection mat, where long contact times between disinfectant and virus are achieved, which means that shoe sole disinfection must always be combined with a rigorous scraping of the shoes on the mat, causing the virus particles from the shoe sole to remain in/on the disinfection mat. In order to be able to make this practice-relevant statement, it is important that the experimental design to be selected also takes practical conditions into account. In this context, it should be noted that the efficacy of disinfectants against (virus) contamination with very short contact times is reported as an important evaluation criterion [35,48]. For shears and other tools, which are only briefly dipped in an appropriate solution, this may certainly be suitable. For other requirements, such as the decontamination of surfaces or the disinfection of shoe soles discussed here, short contact times are not relevant for making practice-relevant product recommendations.

Initially, for the conducted efficacy testing in infected plant homogenate (Table 4) and in the carrier experiment (Figure 3), the literature was screened for products that had already demonstrated high inactivation effects against stable plant viruses, such as tobamoviruses in previous studies, often under in-vitro conditions [35–37,48]. Numerous studies on the antimicrobial mode of action of benzoic acid, the active ingredient in MENNO Florades, have already been reported and were summarized by Chipley [49]. For practical application, it should only be pointed out that the availability of the undissociated molecule of benzoic acid, which is responsible for antimicrobial activity, is related to the pH. As

pH increases, the quantity of undissociated acids decreases, reducing the effectiveness of the active agent [49]. For this reason, the use of a pH-stabilizing buffer in the suspension test (Experiment 1) was avoided. The virucidal effect from sodium hypochlorite and the active ingredient of Virkon S are derived from oxidation activity [50,51], while lactoferrin has a variety of modes of action against different viruses, which are similar in preventing infection of the host cell, e.g., through binding of lactoferrin to the virus particles [52,53]. The approval situation was not considered for these two evaluations.

Similar results regarding the virucidal efficacy of disinfectants against stable plant viruses in a suspension study were reported by Li et al. [35] and Chanda et al. [36]. The high efficacy for inactivating ToBRFV of household bleach products, such as DanKlorix, was confirmed in the suspension test. For skim milk powder, the situation is unclear. Skim milk powder or nonfat-dried milk showed high virus inactivation efficacies, as reported in previous studies [35,54,55]. However, in other studies, nonfat-dried milk did not lead to any deactivation of the infectivity of ToBRFV or of *Cucumber green mottle mosaic virus* (CGMMV), a virus belonging to the same genus [36]. In the suspension test, we could observe a significant reduction in local lesions by applying 1% Virkon S. The high, but not complete, inactivation of stable tobamoviruses by short-term application of 1% Virkon S is also shown by other studies [35,48]. The disinfectant MENNO Florades (4%) also achieved a significant reduction in local lesions. In previous studies on the virucidal activity of MENNO Florades against various plant viruses, a heterogeneous picture was found. In a study by Coutts et al. [40], 3% MENNO Florades did not completely inactivate the potyvirus *Zucchini yellow mosaic virus* with a contact time of 1 min, whereas 2% MENNO Florades (1 min) was able to completely inactivate the tobamovirus CGMMV [37].

Several carrier trials on disinfectant efficacy against phytopathogenic viruses, frequently on tools, have previously been carried out [37,48,56]. This testing methodology has already provided practical knowledge in many fields, which might not have been obtained by pure in-vitro testing [39]. These existing carrier tests are hereby extended to shoe-sole decontamination.

In this experimental design (Experiment 2) with very short contact times of only 30 s, none of the previously tested disinfectants showed any satisfying inactivation of the virus particles, except for Floradix Lactoferin 0.5%. The relatively high efficacy of Floradix against ToBRFV even with short contact times is not surprising; previous studies have already highlighted the antiviral effect of the milk-based glycoprotein lactoferrin [36,52,57,58], which is now confirmed on (rubber) surfaces. The results of the other products contrast strongly with the inactivation effects of disinfectants in ToBRFV-infected plant sap and therefore questions the selected methodology of carrier testing in Experiment 2.

Based on this, we concluded that the simulated decontamination experiment of ToBRFV contaminated carriers, as well as the suspension test, is not suitable for making practical recommendations. This is for various reasons: As noted in previous studies, the inactivating effect of disinfectants in disinfection mats on pathogens is severely limited due to potential shoe contamination and adhesion, as well as an often very short contact time [59], while the correlation of increased contact times and improved effectiveness of horticultural disinfectants has been proven many times [60–62]. Prolonged contact time between virus particles and disinfectant was not achieved in the carrier test, since the factor of mechanical cleaning of the sole by which the contamination entered the disinfection mat, was not taken into consideration. Extended contact times are easy to achieve in such a scenario. Further parameters, such as the pressure on the contaminated sole corresponding to the weight of the person and a realistic discharge behavior could only be fulfilled in the test of the practical shoe decontamination and not in our simulated carrier test. Furthermore, the approval situation of the tested products was not considered. Substances, such as, skim milk powder or lactoferrin indicated high virucidal efficacies but are not approved or licensed to combat plant pathogens in horticultural crop protection. In the case of lactoferrin, high costs are an additional disadvantage. For these reasons, testing shoe-sole decontamination under practical greenhouse conditions is very important.

Considering the depletion of infectious ToBRFV particles from the sole of the shoes in the experiment under practical conditions, it can be concluded that sole disinfection, similar to previous studies [13,63], can contribute to a reduction in the risk of spreading harmful organisms. Such cleaning of the footwear is also possible with water (Figure 4). However, this will create a virus inoculum within the mat from which further spreading can start. The numerous necrotic local lesions on our test plants after inoculation of ToBRFV-contaminated dirt, which were exposed to deionized water (Figure 5) for 4 d, highlights the stability and longevity of tobamoviruses, which has already been demonstrated in previous studies [2,27,64]. Taken together, this indicates an enormous phytosanitary risk in the case of a disinfection mat filled with water, which should be urgently avoided. For this reason, a mat or footbath must be loaded and regularly refilled with an effective and approved disinfectant, such as MENNO Florades. With this study of a three-stage disinfection test on the harmful *Tomato brown rugose fruit virus*, we aimed to demonstrate the need for establishing a standardized test method for scientific investigations in this field to guide tomato growers to approved and effective decontamination strategies.

Besides the efficacy of the product against plant pathogens, such as ToBRFV, additional impacts of the compounds on crop and residues in fruits have to be taken into consideration for any application recommendation [65]. Although the products will not directly be applied to plant or soil, it is feasible in greenhouse tomato production that the crops may be exposed to active ingredients or residues. Previous studies have already shown the non-phytotoxicity of MENNO Florades [65]. When applying MENNO Florades in accordance with application instructions, no relevant effects on the health of consumers were found within the registration report as a plant protectant [66,67]. Moreover, the compound benzoic acid formulated in MENNO Florades is a natural substance in plants that is already widely used as an additive in foodstuffs [67]. From the perspective of the authors, investigations of the impact of potential disinfectants on humans, the environment, plants, and, in the case of greenhouses, on various materials are essential for product recommendations. This aspect should be considered even more in future studies in order to be able to provide ecologically and economically feasible recommendations to apply in practice.

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