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Cleaning of *Tomato brown rugose fruit virus* (ToBRFV) from Contaminated Clothing of Greenhouse Employees

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Abstract: The highly infectious *Tomato brown rugose fruit virus* (ToBRFV) is a new viral threat to tomato production worldwide. In production, the very easy mechanical transmissibility combined with the high resistance in vitro is of great concern. We tested: (i) whether household cleaning products, commercial agricultural detergents, and an authorized plant protectant are suitable for cleaning contaminated clothing, and (ii) whether infectious viruses remain in the resulting cleaning water. The evaluation of the sanitation effect was performed using bioassays, by counting ToBRFV-associated necrotic local lesions on *Nicotiana tabacum* cv. Xanthi NN. For this purpose, leaves were mechanically inoculated with treated fabrics and cleaning solutions which would normally be discharged to the sewer system. The detergents Fadex H^+ (FH) and Menno Hortisept Clean Plus, as well as the disinfectant Menno Florades (MF), led to an almost complete removal of ToBRFV from contaminated fabrics, corresponding to a reduction in local lesions by 99.94–99.96%. In contrast, common household cleaning products (Spee ActivGel (SAG), Vanish Oxi Action Gel (VO) did not effectively remove the pathogen from the fabric, where the reduction was 45.1% and 89.7%, respectively. In particular, cleaning solutions after the use of household cleaners were highly contaminated with ToBRFV. After a 16-h treatment with the disinfectant MF, infectious ToBRFV was no longer present in VO, FH, and MF cleaning solutions, as demonstrated by extensive bioassays.

Keywords: sanitation; fabric; cotton; detergent; emerging measures

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1. Introduction

Solanum lycopersicum L. is one of the world's most important vegetable crops. Tomatoes account for 16% of global vegetable production. The dispersal of viruses in this crop might immediately lead to enormous losses, and therefore it requires the highest degree of precise cultivation management.

Outbreaks of viral diseases, such as *Pepino mosaic virus* (PepMV) or *Tomato brown rugose fruit virus* (ToBRFV), can lead to serious epidemics and cause great financial losses to growers of tomato. PepMV first appeared in glasshouse tomato crops in the Netherlands in 1999 [1] and has rapidly established itself in tomato-producing countries. Yield losses caused by PepMV were estimated to be between 5% and 10%, and they can vary considerably [2]. ToBRFV, a new member of the genus *Tobamovirus*, is an emerging and highly virulent virus. Due to the severe phytosanitary and economic consequences of an outbreak, emergency measures against ToBRFV have been adopted in the EU, while the virus is A1 listed in the UK, Chile, and Argentina [3]. Since the first report of a ToBRFV outbreak in tomato in Jordan [4], the virus has been identified in other countries on different continents [5], and disease incidences up to 100% have been reported [4,6]. Therefore, ToBRFV is considered the most serious threat to tomato production worldwide [7]. Due

to discoloration and rugose, fruits of the infected plants lose their market value or are unmarketable [8]. However, the intensity of symptoms seems to vary according to varieties, management practices, and climatic conditions. In addition, ToBRFV is able to overcome the resistance gene *Tm-2*^{2} [9,10], which is used in tomato hybrids for effective controlling *Tobacco mosaic virus* (TMV) in protected tomato production [11]. Both viruses are localized externally on the tomato seed coat [12,13] and are mechanically transmitted from PepMV- or ToBRFV-contaminated tomato seeds to seedlings, which can initiate a disease focus and eventually lead to further spread in the crop.

In the case of tobamoviruses, the situation is aggravated by the fact these are presumably very stable and retain their infectivity for a long time, even outside the living host plant, e.g., on the surfaces of greenhouse tables, pots, and tools, but also on the fabric worn by employees during cultivation. Since control using curative means is not possible, and resistant varieties have not been available so far, measures such as prophylaxis as well as optimized and fast diagnosis must be applied to prevent further spread and the risk of an infection of complete tomato production [14]. The clothing of farm workers addressed in this study is in frequent physical contact with potentially infected tomato plants during cultivation activities, such as the pruning and tying of tomato plants, as well as during harvesting. If contact occurs between clothing and plants, microlesions can be caused on the plant, allowing ToBRFV particles to be transferred to the clothing and transmitted to a plant the next time it comes into contact with it, potentially causing infection. The risk of spreading the virus through contaminated clothing must be classified as high. A large number of studies on the contamination of clothing or fabrics are available in the area of human pathogens, e.g., in hospitals [15–17]. However, the issue of the decontamination of fabrics used in plant production has hardly been addressed by previous studies. One of the few studies by Broadbent and Fletcher on the epidemiology of the *Tomato mosaic tobamovirus* demonstrated that contaminated clothing poses a significant risk of infection [18]. The authorized agents and procedures for chemo-thermal laundry disinfection for the purpose of preventing and controlling infectious diseases in humans is regulated in Germany in accordance with §18 of the Infection Protection Act [19]. A similar regulation for the requirements of the cleaning and/or disinfection efficiency in the washing process against phytopathogenic organisms does not exist in Germany, and thus it is not regulated. With the emergency measures amended in October 2021 in accordance with (EU) 2021/1809, tomato fruit producers affected by ToBRFV infection have to (i) remove and destroy all specified plants from the production site, at least at the end of the cropping season, and (ii) apply specific hygiene measures on personnel, production site structures, machinery, tools and other materials to prevent the spread of ToBRFV. At the end of the cropping season the removal of the tomato plants as well as the destruction or treatment of the growing medium shall be carried out in such a way that there is no identifiable risk of spreading the virus [20]. Continued cultivation, which might include ToBRFV-infected plants, poses the risk of spreading the pathogen during cultivation and maintenance work both inside the greenhouse and outside the premises. A possible transmission route is not only via tools, but also via work clothing. However, for occupational safety reasons, workers cannot wear disposable safety suits all day in the greenhouse. They wear their normal work clothes, supplemented by disinfectable gloves if necessary. The aim of this study is to clarify whether (i) household laundry products, commercial agricultural detergents, and the authorized plant protectant with a disinfection effect against plant pathogens are suitable for cleaning contaminated clothing, and (ii) if infectious virus particles can still be found in the resulting cleaning water. Cleaning water is normally discharged directly into the sewer system from the washing machine. Here, there would not be an inconsiderable risk of ToBRFV being carried over into the sewage treatment plant or the water treatment system. Due to phytosanitary and regulatory requirements for the inactivation of *Tomato brown rugose fruit virus*, the discharge of the virus from the greenhouse, for instance, by contaminated laundry water, must be prevented. To address these requirements, we tested

the efficacy of the disinfectant Menno Florades to inactivate ToBRFV in cleaning water. Based on the results of the study, we provide recommendations for practical measures.

2. Materials and Methods

2.1. Source of ToBRFV and Plant Material

The ToBRFV isolate PV-1236 was acquired from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and originated from the ToBRFV outbreak in North Rhine-Westphalia in 2018 [21]. The propagation of the pathogen was performed in *Nicotiana clevelandii* A.Gray, which showed a systemic infection. Four weeks after the mechanical inoculation of *N. clevelandii*, the symptomatic leaves were collected and 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.

Bioassays, to confirm the infectivity of ToBRFV in fabric and cleaning solution were conducted with *Nicotiana tabacum* L. cv. Xanthi NN. Test plants were grown in the greenhouse under controlled conditions (20 °C/16 °C day/night and 16 h/8 h light/dark) in pots filled with bedding substrate (Klasmann-Deilmann GmbH, Geeste, Germany). Seeds of *N. tabacum* cv. Xanthi NN were sown in sufficient numbers for each experimental replicate and potted 3–4 weeks after sowing. Another 3 weeks later, the plants were used for the infectivity assays, which took about 1 week each, and were immediately destroyed afterwards. No additional fertilization or biological or chemical plant protection measures were required for the approximately 8-week period from sowing to destruction. All plants were watered manually as needed.

2.2. Inoculum Preparation and Mechanical Inoculation

For the preparation of the inoculum, infected *N. clevelandii* homogenate was ground 1:5 (*w*:*v*) with buffer solution (0.1 M Na_3PO_4, 0.2% Na_2SO_3, pH 7.0) in an extraction bag (Bioreba AG, Reinach, Switzerland), transferred in 50 mL centrifuge tubes (Sarstedt AG & Co. KG, Nürmbrecht, Germany), and kept on ice until use.

Immediately before mechanical inoculation, abrasive non-washed diatomaceous earth [CAS 61790-53-2] was either added to the cleaning solution or dissolved in water and pipetted onto the leaves to be subsequently inoculated with the contaminated fabric (3 × 3 cm 100% cotton work coat; 240 g/m^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.

2.3. Selection of Products and Preparation of the Working Solutions

In total, five different products were tested for the cleaning and decontamination of fabrics (Table 1): the plant protectant Menno Florades (MF) (Menno Chemie-Vertrieb GmbH, Norderstedt, Germany), the detergents Menno Hortisept Clean Plus (HCP) and Fadex H$^+$ (FH) (both Menno Chemie-Vertrieb GmbH, Norderstedt, Germany), and the household heavy-duty detergent Spee ActivGel (SAG) (Henkel Wasch-und Reinigungsmittel GmbH, Düsseldorf, Germany) and stain remover Vanish Oxi Action Gel (VO) (Reckitt Benckiser Deutschland GmbH, Heidelberg, Germany). Every cleaning product, detergent, or disinfectant was prepared with deionized water and adjusted to the required concentration according to the manufacturer's instructions.

Table 1. List of products, their active ingredients, and the tested concentration of the working solution for cleaning artificial *Tomato brown rugose fruit virus* (ToBRFV)-contaminated fabrics.

Product	Active Ingredient		Working Solution	
Trade name	**Name**	**Concentration**	**Concentration**	**pH**
Menno Florades (MF)	Benzoic acid	9% (*w*/*v*)	4% (40 mL/L)	2
Menno Hortisept Clean Plus (HCP)	Sodium hydroxide	10− < 15%	2% (20 mL/L)	12.5
Fadex H^+ (FH)	Formic acid	45− < 50%	2% (20 mL/L)	1
Spee ActivGel (SAG)	Anionic tensides	5–15%	0.25% (2.5 mL/L)	5
Vanish Oxi Action Gel (VO)	Hydrogen peroxide	≤10%	2.5% (25 mL/L)	6

2.4. Experimental Design

We simulated a simple and rapid prewash process to demonstrate the cleaning potential of various products with respect to *Tomato brown rugose fruit virus*-contaminated clothes. We addressed three questions: (i) Do contaminated clothing that have been pre-washed still pose a risk of spreading ToBRFV (fabric), (ii) does the prewash detergent (cleaning solution) still contain infectious ToBRFV particles, and (iii) does the addition of the disinfectant Menno Florades (4%, contact time 16 h) to the contaminated cleaning solution ensure a reliable inactivation of ToBRFV.

The experiment was performed as a fully randomized study under controlled conditions in the greenhouse in 2021–2022 and was repeated three times. A standardized three-step process was used to perform: (1) contamination of working clothes, (2) cleaning of contaminated working clothes, and (3) proof of sanitation via bioassay (Figure 1). First, the ToBRFV-containing plant sap was prepared as described in 2.2. Afterwards, the cloth carriers (3 cm × 3 cm) were contaminated with 500 µL infected plant sap each, and allowed to stand for approximately two hours. One contaminated cloth carrier each was placed in a 50 mL centrifuge tube filled with 20 mL of the respective detergent solution and immediately placed on a mini-shaker for 10 min (1000 rpm) at 20 °C room temperature. Particular care was taken to ensure that the entire cloth carrier remained in the solution. After 10 min contact time, the carrier was taken out of the solution and pressed out. The carrier was then inoculated onto three leaf halves per test plant, which were rubbed with non-washed diatomaceous earth in advance to create lesions. At the same time, 100 µL each was taken from the remaining cleaning water solution and mixed with 900 µL of deionized water and a defined amount of non-washed diatomaceous earth in a 2 mL reaction tube. Then, three leaf halves of a test plant were inoculated with 150 µL per reaction tube. The solution was pipetted onto the leaf halves and evenly distributed with the fingers. For a further test, 960 µL of the cleaning water was taken again and added to a 2 mL reaction vessel. To each of these, 40 µL of the disinfectant Menno Florades was added and allowed to stand for 16 h. Only the two variants, "ToBRFV sap" and "MF 4 h" did not receive any additional Menno Florades. After the 16-h contact time, 100 µL were taken from each reaction tube and placed in a 2 mL reaction tube filled with 900 µL of deionized water and non-washed diatomaceous earth, and mixed. A total of 150 µL solution was pipetted onto each of the three leaf halves of the test plant, and evenly distributed with the fingers.

In total, three leaf halves were inoculated on each of eight plants per repetition and treatment, resulting in 24 individual scores for each treatment repetition. Therefore, the obtained results of this study are based on a total of 504 inoculated plants with 1512 inoculated leaf halves. In addition to the inoculated plant/leaf halves, three plants of *N. tabacum* cv. Xanthi NN were mock-inoculated per treatment, and served as a negative control that was exclusively treated with the putative products only, but not with the virus particles. The positive control was always represented by "Control" treated with deionized water. To evaluate the results of the cleaning solution, as well as the cleaning solution treated with Menno Florades, it should be mentioned that the inoculated solutions were obligatorily diluted 1:10 in deionized water, so that phytotoxic damage to the inoculated leaf halves, induced by the detergents and disinfectants, could be prevented or mitigated.

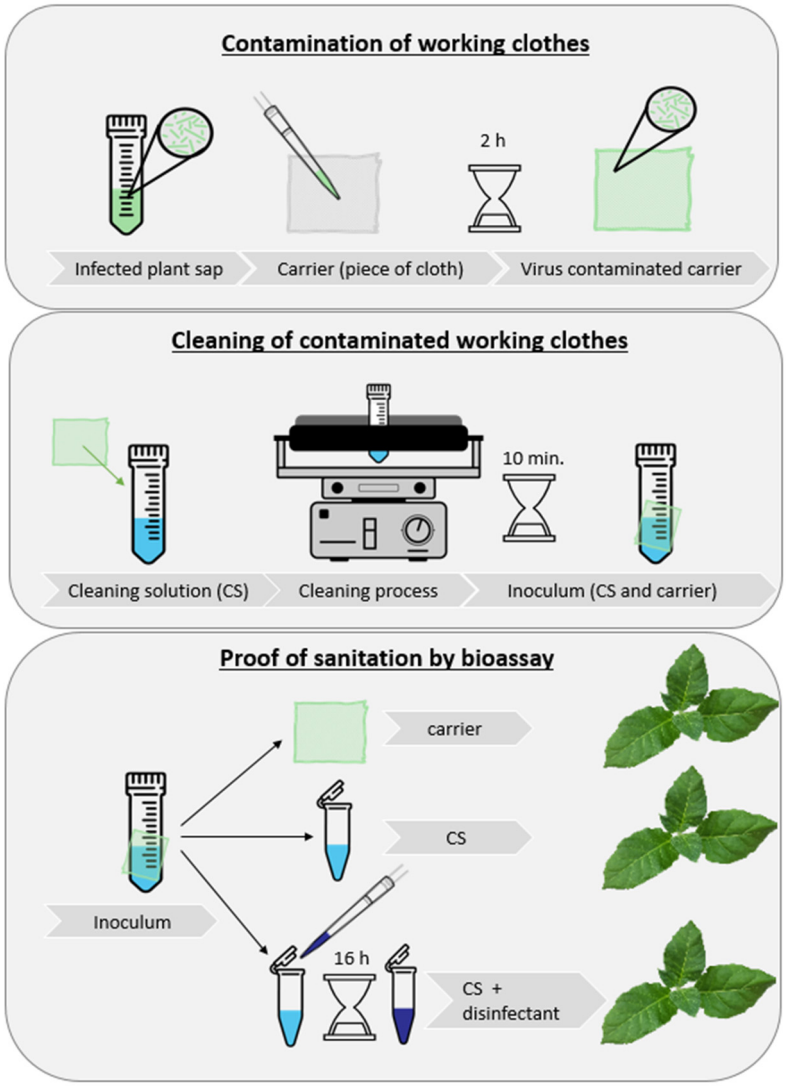

Figure 1. Methodical approach of cleaning *Tomato brown rugose fruit virus* (ToBRFV)-contaminated clothing and proof of sanitation via bioassay on *Nicotiana tabacum* cv. Xanthi NN.

2.5. Detection of ToBRFV

For the bioassay, the susceptible host plants *N. tabacum* cv. Xanthi NN were mechanically inoculated and visually evaluated 6–7 days after inoculation (dai) by determining the number of necrotic lesions per inoculated leaf half. Infected plants showed characteristic necrotic local lesions, which have already been described for tobamoviruses on species of *Nicotiana* [22] (Figure 2). The methodology of counting necrotic local lesions was recently used to evaluate the efficacy of various disinfectants on ToBRFV-contaminated razor blades, using the indicator plants *N. rustica* L. and *N. benthamiana* L. [23]. The necrotic local lesions were detectable 3 to 4 days post-inoculation, first as necrotic spots (<0.5 mm), and could be clearly distinguished from any mechanical damage or phytotoxic effects of the tested products by light-colored necrotic leaf tissue in the center, surrounded by a slightly darker brown edge. At 6–7 days after inoculation, a diameter of up to 4 mm for the necrotic local lesions facilitated the scoring.

Figure 2. Necrotic local lesions on N. tabacum cv. Xanthi NN (in this case, 105 local lesions were counted) following mechanical inoculation of one leaf half with Tomato brown rugose fruit virus (ToBRFV).

Composite samples of the inoculated leaf halves were taken, and they underwent a double antibody sandwich enzyme-linked-immunosorbent assay (DAS-ELISA) to confirm an infection with ToBRFV, and to clearly classify that the observed local lesions are caused by ToBRFV and do not represent phytotoxic damage. DAS-ELISA was performed using a commercially available assay (RT-1236) according to the suppliers' instructions (DSMZ, Braunschweig, Germany) [24]. Deviating from the protocol, a 1:10 (w/v) extraction buffer was used instead of a 1:20 (w/v) extraction buffer. Each sample was tested with at least two replicates. The optical density (OD) of the samples at 405 nm was rated after 60 min substrate incubation. 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 being ToBRFV-positive.

2.6. Data Analysis

Statistical analysis was performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

Infection status of the 24 plants per treatment was arranged in two-dimensional cross-tabulations [treatment (products tested) × infection (no/yes)], and pairwise comparisons of treatments with respect to the proportion of infected plants were assessed in 2 × 2 tables (as part of the total table) using Fisher's exact test procedure with Bonferroni correction (SAS procedure FREQ).

The number of lesions was summarized from leaf halves resulting in 24 observations per treatment (8 plants × 3 trial replications). A one-factorial model with products as fixed treatment factor, assuming a negative binomial distribution for the residuals, was used to analyze these count data (zero count values were replaced by 0.01). The analysis was performed within a framework of a generalized linear mixed model (GLMM; SAS procedure GLIMMIX) [25].

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. Infectivity of Tomato Brown Rugose Fruit Virus in N. tabacum cv. Xanthi NN

The infectivity of the inoculum was maintained after cleaning if necrotic local lesions appeared reliably on the inoculated leaf halves on the test plant *N. tabacum* cv. Xanthi NN, 6–7 dai. DAS-ELISA confirmed the ToBRFV infection of all tested leaves, with necrotic local lesions considered to be infected based on bioassay. The number of expressed local lesions of the dilution 1:5 (*) equals the inoculum that was used to contaminate the fabrics (Figure 3). The next dilution level (**) corresponds to the virus dilution found in the cleaning solutions, assuming that all ToBRFV particles could be dissolved out of the contaminated fabric. The third dilution level (***) represents the virus concentration that was actually inoculated on the leaves as cleaning solutions, assuming that all ToBRFV particles were removed from the fabric and no inactivation of ToBRFV took place. The dilution series showed that the number of necrotic local lesions decreased from dilution to dilution, but the reduction was not linear. While in the first two dilution steps the reduction in expressed local lesions on the test plants was approximately 25%, in the following steps, the percentage reduction increased from approximately 40% up to 90%.

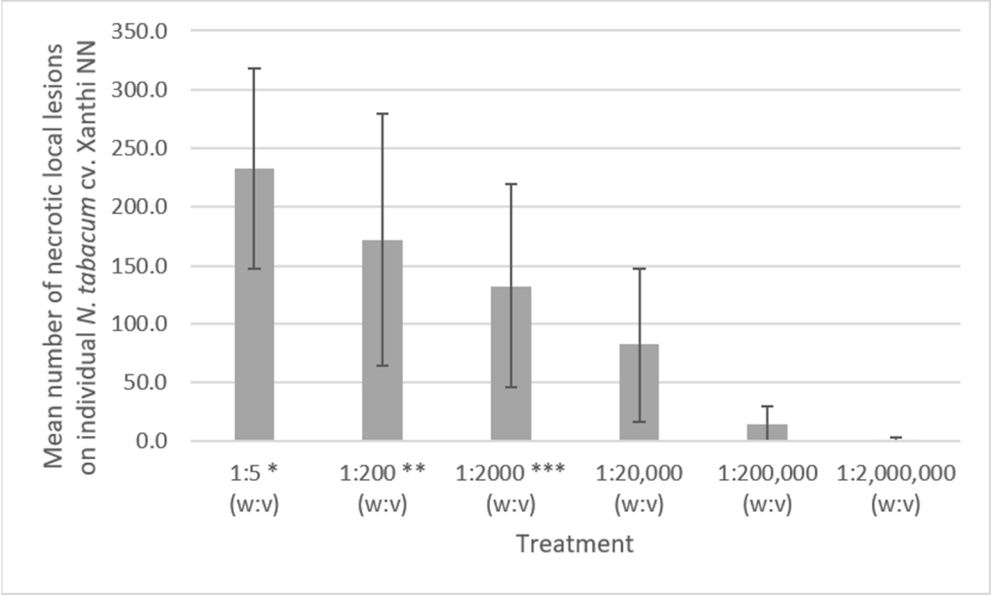

Figure 3. Mean number of necrotic local lesions on individual *N. tabacum* cv. Xanthi NN plant after mechanical inoculation of a dilution series, based on the inoculum *Tomato brown rugose fruit virus* (ToBRFV) sap used (infected *N. clevelandii* leaf:buffer 1:5 (*w*:*v*), up to 1:2,000,000 (*w*:*v*). n = 10. * equals the inoculum that was used to contaminate the fabrics; ** equals to the virus concentration found in the cleaning solutions, assuming that all ToBRFV particles could be dissolved out of the contaminated fabric; *** represents the virus concentration that was inoculated on the leaves as cleaning solutions, assuming that all ToBRFV particles were removed from the fabric and no inactivation of ToBRFV took place.

3.2. Qualitative Cleaning Efficacy of Detergents and the Disinfectant

To determine the remaining infectivity on fabric and in the cleaning solution, respectively, after treatment with the respective product, mechanical rub-inoculation was performed, as described in previous studies [26–28]. Infection of the test plants was declared when at least one necrotic local lesion occurred on one of the three inoculated leaf halves. In the study, mechanical rub-inoculation of contaminated fabric and cleaning so-

lution resulted in 100% infestation when treated exclusively with ToBRFV-infected plant sap, and in a 0% infestation rate in mock-inoculated variants (negative control) (Table 2). MF 4% resulted in significant cleaning and/or inactivation of infectious ToBRFV particles on contaminated clothing, in particular, with a contact time of 4 h. In addition to Menno Florades, the two tested detergents HCP and FH, both 2%, achieved a high cleaning efficacy. For the two household laundry products SAG 0.25% and VO 2.5%, 24 out of 24 plants were infected in each case. Accordingly, no cleaning of the stable tobamovirus was achieved by these products.

Table 2. Cleaning efficacy of different products on *Tomato brown rugose fruit virus* (ToBRFV)-contaminated clothing determined on the clothing itself (cloth carrier) and the cleaning solution (CS), using bioassays on *N. tabacum* cv. Xanthi NN. If any plant had at least one ToBRFV-associated necrotic local lesion, the plant was scored as infected. Letters based on pairwise treatment comparisons using Fisher's exact test (alpha = 0.05, with Bonferroni correction).

Treatment	Cleaning Time	Cloth Carrier	Cleaning Solution (CS)
number of infected plants/total number of plants			
Negative control	10 min.	0/15	0/15
Control	10 min.	24/24 (A)	24/24 (A)
Disinfectants			
MF 4%	10 min.	5/24 (B)	7/24 (B)
MF 4%	4 h	2/24 (B)	8/24 (B)
Detergents			
HCP 2%	10 min.	2/24 (B)	1/24 (B)
FH 2%	10 min.	3/24 (B)	1/24 (B)
Household detergents			
SAG 0.25%	10 min.	24/24 (A)	24/24 (A)
VO 2.5%	10 min.	24/24 (A)	24/24 (A)

'Control' = Fabric/CS contaminated with ToBRFV sap; SAG' = Spee Activ Gel; 'VO' = Vanish Oxi Action Gel; 'MF' = Menno Florades; 'FH' = Fadex H^+; 'HCP' = Hortisept Clean Plus.

The results of the cleaning procedure clearly indicate that commercially available laundry products do not sufficiently remove ToBRFV from fabric. Furthermore, the resulting cleaning solutions of these products pose a significant carryover risk of ToBRFV. For this reason, we demonstrated the ability of the disinfectant Menno Florades (4%, contact time 16 h) to decontaminate these cleaning solutions (Table 3).

The application of Menno Florades resulted in an almost complete inactivation of ToBRFV, especially in the previously highly contaminated cleaning solutions of SAG and VO. The MF 4% 10 min variant received a further addition of Menno Florades after the cleaning step. In this case, complete inactivation was achieved. Only for the HCP cleaning water was no further inactivation effect observed via the application of Menno Florades, when compared to the results of the cleaning solution from Table 2. A more detailed evaluation of the cleaning and inactivating effect of the cleaning and disinfecting products was conducted in addition to the plain qualitative statement (yes/no), by choosing the test plant *Nicotiana tabacum* cv. Xanthi NN. That cultivar shows characteristic countable necrotic local lesions after a ToBRFV infection. For reasons of simplicity, the standard error of mean (SEM) is not shown in the following graphs. However, this information is provided in the Supplementary Material Table S1: Lesions (plant) pairwise.

Table 3. Inactivation efficacy of the disinfectant Menno Florades on *Tomato brown rugose fruit virus* (ToBRFV)-contaminated cleaning solutions measured using bioassays on *N. tabacum* cv. Xanthi NN. If any plant had at least one ToBRFV-associated necrotic local lesion, the plant was scored as infected. Letters based on pairwise treatment comparisons using Fisher's exact test (alpha = 0.05, with Bonferroni correction).

Cleaning Solution (CS)	Disinfectant	Disinfection Time	Decontaminated CS
number of infected plants/total number of plants			
Negative control	MF 4%	16 h	0/15
Control	/	/	24/24 (A)
Disinfectants			
MF 4% 10 min	MF 4%	16 h	0/24 (B)
MF 4% 4 h	/	/	2/24 (B)
Detergents			
HCP 2%	MF 4%	16 h	3/24 (B)
FH 2%	MF 4%	16 h	0/24 (B)
Household detergents			
SAG 0.25%	MF 4%	16 h	2/24 (B)
VO 2.5%	MF 4%	16 h	0/24 (B)

'Control = Deionized water with ToBRFV-contaminated fabric; SAG' = Spee Activ Gel; 'VO' = Vanish Oxi Action Gel; 'MF' = Menno Florades; 'FH' = Fadex H^+; 'HCP' = Hortisept Clean Plus.

3.3. Cleaning of ToBRFV-Contaminated Clothes

It can be stated that the methodology of mechanical rub-inoculation of the cloth piece on the test plants *N. tabacum* cv. Xanthi NN resulted in the reliable expression of necrotic local lesions. For the Control variant, in which the ToBRFV-contaminated cloth pieces were washed exclusively in deionized water, approximately 450 lesions per test plant were counted (Figure 4). The cleaning process using the respective products resulted in a significant decrease in ToBRFV-associated necrotic local lesions for all products, except for SAG. It can be assumed that the ToBRFV-containing plant sap was washed out of the fabric, and therefore, the virus particles were also removed. The products evaluated may be divided into two categories. On the one hand, there are household detergents such as SAG and VO, which did not result in reliable cleaning efficiency, indicating the possibility of spreading ToBRFV despite cleaned clothing, and on the other hand, agricultural detergents and disinfectants, such as HCP, FH, and MF, which resulted in an almost complete removal of infectious ToBRFV from contaminated fabric. While the cleaning steps with SAG led to a 45.1% reduction in local lesions, and with VO, a reduction of 89.7% of local lesions, the agricultural products HCP, FH, and MF achieved a reduction in local lesions by 99.94–99.96% when compared to "Control". No significant differences were detected among the agricultural products.

Referring to the cleaning efficiency corresponding to cleaning solutions on the cloth carriers, negative effects such as phytotoxic damage to the test plants and damage to the cloth carriers were also taken into consideration. No visible damage to the fabric textile caused by the cleaning process or the tested product was detected for any of the used fabric pieces. However, further investigations are necessary for reliable conclusions on the effects by cleaning solutions on the material. It was generally observed that all tested cleaning solutions in the tested concentrations, which were present in the fabric to be inoculated, had good plant tolerance, with the exception of FH. Fadex H^+ containing the washing ingredient formic acid at an application concentration of 2%, which resulted in extensive necrosis a few seconds after contact with the test plants, followed by death of the inoculated leaves. To prevent leaf damage, leaves were sprayed with water a few seconds after inoculation.

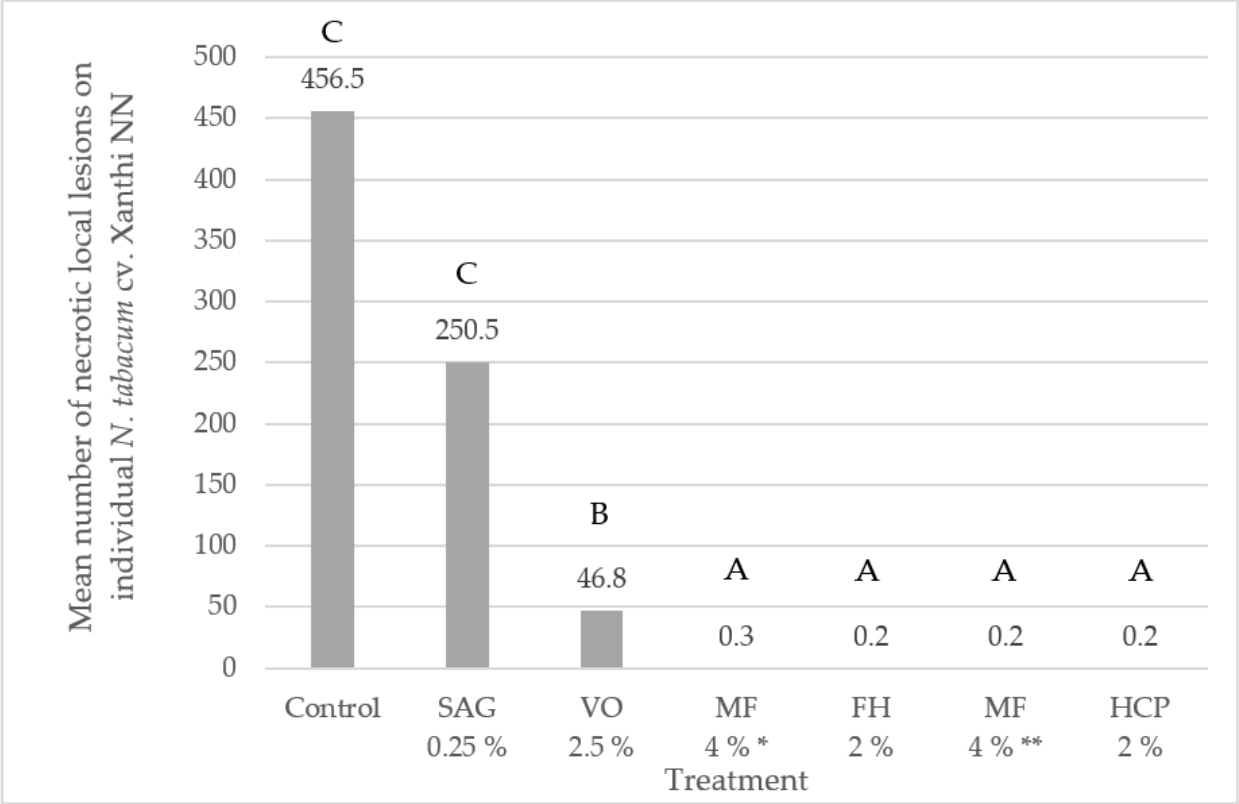

Figure 4. Evaluation of the cleaning efficacy of different products on *Tomato brown rugose fruit virus*-contaminated clothes by counting the number of necrotic local lesions on individual *N. tabacum* cv. Xanthi NN plants that were rub-inoculated with the treated (cleaned) cloth carriers. Letters based on pairwise treatment comparisons using generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction). n = 24. 'Control' = Fabric contaminated with ToBRFV sap; 'SAG' = Spee Activ Gel; 'VO' = Vanish Oxi Action Gel; 'MF' = Menno Florades; 'FH' = Fadex H^+; 'HCP' = Hortisept Clean Plus. * 10 min contact time; ** 4 h contact time.

3.4. Infectivity of ToBRFV in the Resulting Cleaning Solution

The cleaning solution resulting from the cleaning process was clearly contaminated with ToBRFV in the case of the application of household laundry products (Figure 5). Therefore, cleaning solutions that are not decontaminated after the washing process pose a great risk of spreading the harmful organism. As expected, the contaminated cleaning solution caused more local lesions than the fabric piece after exposure to VO. It can be assumed that ToBRFV was removed efficiently from the contaminated fabric. The reduction in necrotic local lesions was only 7.2% and 49.6% for SAG and VO, respectively, in contrast to the deionized water with ToBRFV-contaminated fabric (Control). In contrast, the products HCP, FH, and MF again showed high efficacy, as demonstrated in fabric cleaning. An extension of the contact time of the disinfectant Menno Florades from 10 min to 4 h further reduced the number of induced local lesions. A cleaning procedure with these agricultural products resulted in a significant reduction in local lesions, of between 99.4% and 99.9%.

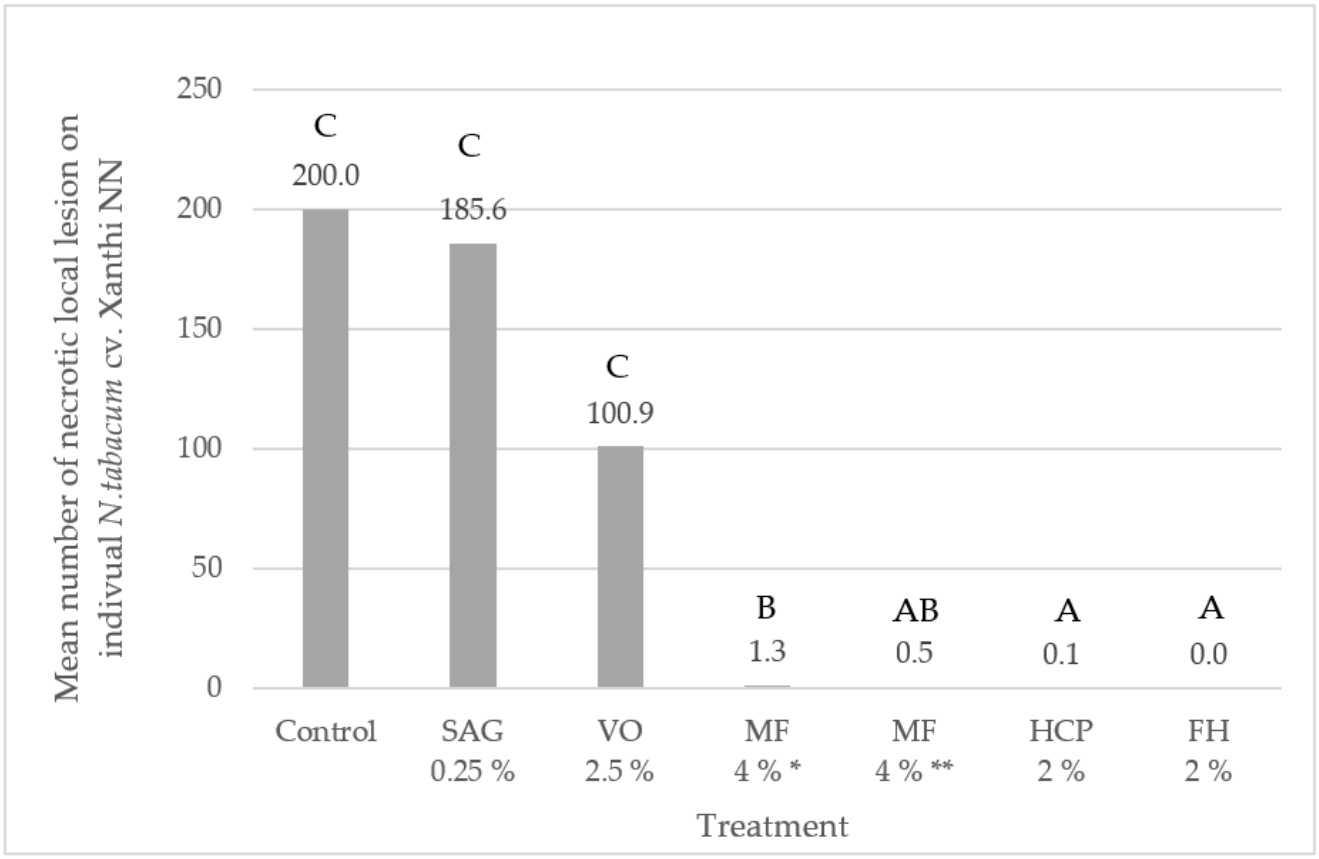

Figure 5. Evaluation of the residual infectivity of *Tomato brown rugose fruit virus* in the various cleaning solutions generated during the cleaning of ToBRFV-contaminated clothing by counting the number of necrotic local lesions on individual *N. tabacum* cv. Xanthi NN plants. Letters based on pairwise treatment comparisons using a generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction). n = 24. 'Control' = Deionized water with ToBRFV-contaminated fabric; 'SAG' = Spee Activ Gel; 'VO' = Vanish Oxi Action Gel; 'MF' = Menno Florades; 'FH' = Fadex H^+; 'HCP' = Hortisept Clean Plus. * 10 min contact time; ** 4 h contact time.

Since the contaminated cleaning solution was diluted 1:10 with deionized water prior to inoculation, no phytotoxic damage to plants was observed for any of the products.

3.5. Disinfection of the Contaminated Cleaning Solution

It has been demonstrated that contaminated fabric that has undergone pre-washing poses a risk of spreading ToBRFV, and also that the resulting cleaning solution is considered to cause problems due to phytosanitary perspective. To prevent the risk of spreading ToBRFV by contaminated cleaning solutions, the corresponding solutions, with the exception of "Control" and "MF 4 h", were treated with the disinfectant Menno Florades (4%) for 16 h (Figure 6). After the application of MF, an almost complete inactivation of ToBRFV could be obtained in all contaminated cleaning solutions. Even the SAG and VO cleaning solutions, which were heavily contaminated before application, showed almost no local lesions after decontamination with MF. The significant reduction in necrotic local lesions when using MF compared to the deionized water with ToBRFV-contaminated fabric (Control) in the different cleaning solutions was 99.5–100%. Based on these results, it can be concluded that the application of the disinfectant Menno Florades (4%) with incubation time of 16 h as recommended by the manufacturer obtains a reliable inactivation of ToBRFV. No phytotoxic damage was observed on the test plants by corresponding detergents or disinfectants.

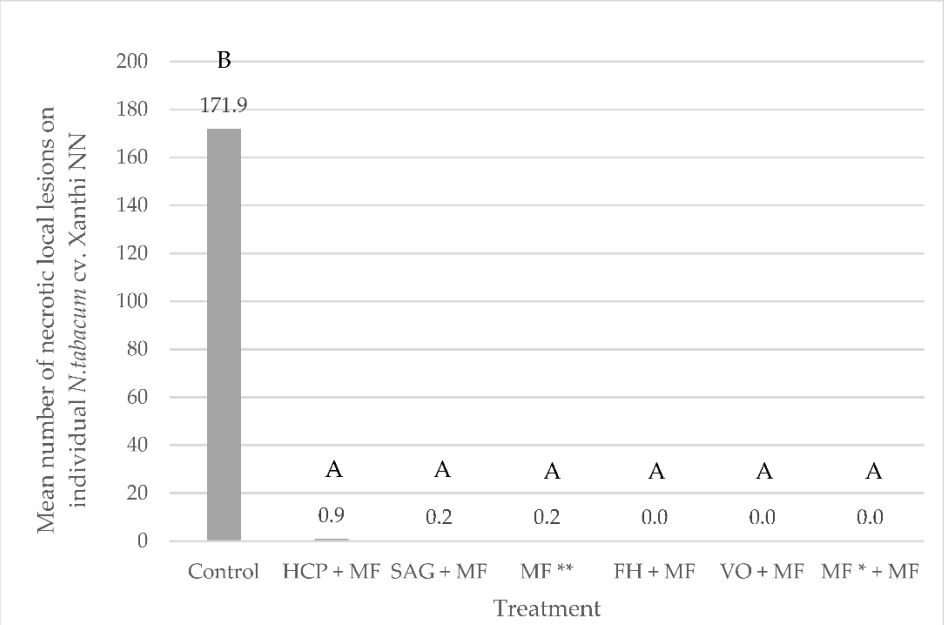

Figure 6. Evaluation of the residual infectivity of *Tomato brown rugose fruit virus* in the various cleaning solutions generated during the cleaning of ToBRFV-contaminated clothing after the addition of the disinfectant Menno Florades (4%) for 16 h by counting the number of necrotic local lesions on leaf halves of *N. tabacum* cv. Xanthi NN. Letters based on pairwise treatment comparisons using generalized linear mixed model analysis of count data (alpha = 0.05, with Bonferroni correction). n = 24. 'Control' = Deionized water with ToBRFV-contaminated fabric; 'SAG' = Spee Activ Gel; 'VO' = Vanish Oxi Action Gel; 'MF' = Menno Florades; 'FH' = Fadex H^+; 'HCP' = Hortisept Clean Plus. * 10 min contact time; ** 4 h contact time.

4. Discussion

The *Tomato brown rugose fruit virus* poses a major threat to tomato production. The spread of the virus in countries of importance for tomato production [21,29–32] is most likely via infected seeds [27]. The lack of resistance genes in commercial tomato cultivars [9] exacerbates the enormous potential for losses. At the same time, experience from previous outbreaks has shown that entire crop stands can be affected, most likely due to the easy mechanical transmission of ToBRFV [4,6,33,34]. Disease control and an optimized management system form the basis for future stable tomato production.

The total world tomato production in 2019 was approximately 180 M tons [35]. In Europe, approximately 16.5 M tons were produced in 2020, of which approximately 10 M tons were used for processing [36]. Greenhouse tomato production has increased significantly over the last decade. Suitable growing conditions and their monitoring, combined with proper maintenance techniques, allows for off-season year-round production. Such an intensive production yields approximately 15 times more per surface unit of measure than under field conditions, and more than 90% of the tomato fruits are marketable, compared to 40–60% under field production [37]. Cultural practices, in particular hydroponics, enable the resource-saving handling of water, but the recirculation of fertigation water creates excellent conditions for a number of stable plant viruses. These can keep infectivity in irrigation and fertigation water, as well as on surfaces, and are mostly easily transmitted mechanically. Likewise, intensive hands-on activities further promote the distribution of plant viruses in the crop. A study on the epidemiology of ToBRFV in tomato greenhouses showed a strong increase of the proportion of ToBRFV infected tomatoes after starting the harvest and following cultivation activities [6].

Multi-layered management to interrupt transmission pathways is mandatory in the control of stable viruses. In addition to seeds and plants, substrates, irrigation water, tools, implements, storage and transport facilities are known to pose a risk of transmission. We

have turned our attention to the hitherto unexamined topic of work clothing. In view of the fact that disposable clothing is hardly ever used in practice, we set ourselves the task of (i) assessing the risk resulting from the cleaning of potentially contaminated clothing and (ii) identifying possible solutions for limiting the risk.

During leaf and fruit sampling in a ToBRFV-infested greenhouse in Germany, the authors of this study were able to demonstrate that the single-use jumpsuit worn became sufficiently highly contaminated to transmit ToBRFV to non-infected test plants after approximately 30 min of wear in the infected crop. The result was serologically confirmed using a DAS-ELISA. This finding highlights the fact of spreading ToBRFV by contaminated work clothing in greenhouses and encourages awareness of the tremendous importance to consider hygienic measures leading to a successful integrated pest management strategy [38].

In this study, we inoculated fabric carriers with *Tomato brown rugose fruit virus*-contaminated plant sap from infected plants to simulate the scenario of an infected tomato leaf and/or fruit being rubbed on the clothing of the worker. Based on the results of the study, we can conclude that a 10-min prewash step at 20 °C with deionized water and household laundry detergents does not remove *Tomato brown rugose fruit virus* sufficiently, neither in the fabric nor in the resulting contaminated cleaning solution, which can cause a great risk of further infections. The heavy-duty detergent Spee Activ Gel contains anionic surfactants, which gives the product its cleaning properties through a variety of different mechanisms. It can be stated that the surfactants reduce the surface tension of the water or the interfacial tension between water and other phases, and therefore facilitates the removal of contaminants from the fabric [39]. This mode of action was visually confirmed by the fact that the resulting cleaning solution was significantly more greenish colored from the extracted plant sap than the control variant, which was washed exclusively with deionized water. The high number of necrotic local lesions for the SAG cleaning solution (Figure 5) highlights the effect, but also illustrates the enormous risk of ToBRFV carryover from contaminated fluids. However, compared to Spee Activ Gel, the other household product, Vanish Oxi Action Gel, based on hydrogen peroxide, showed a significant, although not sufficient, reduction in ToBRFV-associated necrotic local lesions. Compared to other bleaching agents, hydrogen peroxide has the advantage of being environmentally friendly, causing no corrosive damage, and having significantly less fiber damage to clothing [39]. Assuming that the clothing worn in daily practice is significantly less contaminated than the fabric carriers used in this study, a prewashing step with a product containing hydrogen peroxide (VO) might reduce contamination, but with unknown varying rest contamination. At the same time, increasing the temperature of the cleaning solution to above 50 °C can result in a higher efficacy of the washing agent [39]. If there is a suspicion of ToBRFV, the use of these products is strongly discouraged. Nevertheless, if these household laundry detergents are used exclusively, the contaminated cleaning solution must be treated with Menno Florades to prevent the spread and discharge of the virus (Figures 5 and 6).

In Europe, disinfectants for plant disease control are regulated as crop protection products and require approval. Menno Florades is such an approved plant protection product to control, among others, phytopathogenic viruses such as ToBRFV. Previous studies on the efficacy of Menno Florades against stable plant viruses show, as do our results, that the reliable inactivation of tobamoviruses can be achieved by applying 4% Menno Florades [40]. In addition to its good efficacy in terms of cleaning contaminated clothing and decontaminating solutions containing ToBRFV, the active ingredient benzoic acid, which occurs naturally in plant tissue, poses a relatively low toxicity and is considered to be relatively harmless when used according to instructions, especially when the active ingredient is not ingested, e.g., through food [41–43]. If clothes that are contaminated with ToBRFV undergo a prewash with Menno Florades 4%, Fadex H^+ 2%, or Menno Hortisept Clean Plus 2% for 10 min, a large amount of the virus particles can be removed or inactivated.

Compared to given references, the two detergents Fadex H^+ and Menno Hortisept Clean Plus were tested for the first time in regard to their cleaning efficacy on contaminated clothing. Therefore, the promising results obtained cannot be cross-checked with results from the literature. The washing agent formic acid contained in Fadex H^+ is primarily used to control infectious diseases, e.g., in poultry production [44]. In the studies shown here, the very strongly acidic (pH 1) cleaning solution resulted in the almost complete removal of ToBRFV, both from the clothing and in the solution.

The other detergent used in the experiment, Menno Hortisept Clean Plus, contains sodium hydroxide (NaOH). Studies on the efficacy of this substance against other tobamoviruses such as *Yellow tailflower mild mottle virus* (YTMMV) were able to demonstrate that NaOH leads to a partial inactivation of the tobamoviruses [45,46].

Another important aspect in a comprehensive greenhouse sanitation protocol is the decontamination of the cleaning water, especially the water used in the laundering process. According to the results of the dilution series of ToBRFV-infected sap, necrotic local lesions still develop even when the infected plant sap is diluted with deionized water by 1:2,000,000 (*w*:*v*). In relation to practice, this means that all solutions and wastewaters generated in everyday production can potentially lead to an infection, and consequently, to the spread of ToBRFV, both inside and outside the farm.

This study demonstrated reliable inactivation of *Tomato brown rugose fruit virus* in cleaning solutions could be achieved by using the plant protectant Menno Florades (4%) for 16 h in the respective solution. In particular, the inactivation of ToBRFV by Menno Florades becomes evident in the cleaning solutions SAG and VO, which were heavily contaminated prior to application. For the cleaning solutions FH, VAO, and MF 10 min., which were subsequently treated with Menno Florades, complete inactivation of ToBRFV was observed. In the evaluation of the inactivation effect of Menno Florades, the HCP solution stands out as showing the highest number of necrotic local lesions in this study, although this detergent was the most effective in removing ToBRFV from the fabrics. The lack of an inactivating effect of MF can most likely be explained by the fact that the cleaning solution is strongly alkaline (pH 12.5). By adding and adjusting a 4% Menno Florades concentration, the pH of the solution was lowered to approximately 10, which was clearly higher than the pH value of 4.5 specified by the manufacturer, below which dipping solutions should be used for the optimum effectiveness of MF. At higher pH values, the effectiveness of benzoic acid is reduced due to the strong decrease in the quantity of undissociated acid [42]. These results demonstrate that an effective and approved disinfection product is available to growers, but when applying the product, the pH of the solution to be treated must always be considered. The results and recommendations of the present study are addressed in particular for practical use as there are, e.g., smaller companies that carry out their laundry operations on site. In contrast large companies with dozens or hundreds of workers will find it difficult to integrate such pre-treatment of work clothing into their regular operations and ensure its implementation, but this will be a future task. In this case, certified professional textile services according to the quality mark RAL-GZ 992 would come into question [47]. Those companies apply registered and tested thermal or chemo-thermal disinfection procedures, serving hospitals and home care residents. The effectiveness of the disinfection processes is regularly tested on site in the individual laundries using thermologgers and test germs that are harmless to humans. However, to our knowledge, the cleaning processes have not yet been tested with a view towards their suitability to inactivate plant pathogens such as ToBRFV and thus reduce the risk of germ transmission.

In addition to evaluating the cleaning efficacy of several products based on the example of ToBRFV on clothing, this investigation can also be considered as a basis for the development of future practical cleaning standards in the field of phytopathogenic organisms. As has already been mentioned, such testing standards already exist in other fields of application [48,49] and especially in human medicine [50], but they are completely absent in the one considered here. However, comparable and reproducible efficacy evaluations can

only be performed if the requirements for the studies are clearly specified and accessible, e.g., in terms of design, approach, and evaluation.

With this study, the basis will also be laid for a stronger focus on humans and their role as distributors of mechanically transmitted plant-damaging organisms in the future. In the research area of disinfectant testing on *Tomato brown rugose fruit virus*, studies have been conducted to date, on seed coats [27,51], soil [52] and in vitro [53]. Studies on the cleaning and/or disinfection of clothing, shoes, gloves, or even the skin from ToBRFV were missing so far, although its importance for the spread of ToBRFV in greenhouses has already been pointed out [54]. This study provides a big footstep towards effective hygiene management.

5. Recommendations for Practical Application in Greenhouses

Only by implementing an effective hygiene strategy, ranging from virus-free seeds/seedlings to the effective disinfection measures of surfaces, tools and sewage, to the effective cleaning of the clothing of farm workers, it is possible to prevent or reduce the risk of introduction and spread of ToBRFV in tomato crops.

Referring to our studies, *Tomato brown rugose fruit virus*-contaminated work clothing worn by farm employees can contribute to the spread of the virus in the greenhouse, posing a major economic and phytosanitary risk to tomato production. A quick washing step with the household heavy-duty detergent Spee Activ Gel and the stain remover Vanish Oxi Action Gel results in no or only insufficient removal of ToBRFV from the fabric, and are therefore unsuitable if there is a concrete suspicion of ToBRFV or confirmed infestation. The extent to which a longer residence time in the cleaning solution and higher washing temperature could lead to a better washing result remains to be investigated. Regardless of this, it must be assumed that the viruses removed from the fabric remain in the cleaning solution and are infectious. As shown, the cleaning solutions resulting from the two household products presented a high level of contamination of infectious ToBRFV particles, and for this reason, they should never be disposed untreated in the sewerage system. In contrast to these products, the agricultural detergents used, Menno Hortisept Clean Plus, and Fadex H^+, as well as the plant protectant Menno Florades, were able to achieve the safe cleaning of ToBRFV from clothing. Almost no infectiousness could be detected from the formerly contaminated fabric after 10 min of contact. Similarly, the corresponding cleaning solutions posed almost no risk of contamination from the ToBRFV particles washed out. Therefore, in the case of a concrete suspicion of ToBRFV, it is recommended to first pre-treat the worn clothing with the professional cleaning and plant protection products under investigation and only then to subject it to conventional cleaning in a washing machine. In this context, it is important to mention that in the presented study, no negative effect of any product on cotton clothing was observed. However, if it is not possible to treat the clothing with these products, the resulting cleaning solutions should still be compulsorily collected and treated with the disinfectant Menno Florades (4%) for 16 h for a reliable inactivation of *Tomato brown rugose fruit virus*, to prevent further spread of the harmful organism.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8080751/s1, Table S1: Lesions (plant) pairwise.

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