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Paths of cucumber green mottle mosaic virus disease spread and disinfectant-based management

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Abstract

Cucumber green mottle mosaic virus (CGMMV) assigned to the genus *Tobamovirus* is considered a major disease cause of cucurbits worldwide. A primary route for CGMMV disease spread is via mechanical contact. The virus is highly stable and adheres to various agricultural equipment. In the current study, we examined means to inactivate the virus and reduce disease spread via planting equipment and supplies using various chemicals. We have found that incubations of CGMMV-infected cucumber plant extracts with MENNO-Florades 2%, Virocid 3% or Green Up D 20% inactivated the virus and prevented disease spread in a biological assay. Stabilised chlorine formulation (KlorBac), which has the active ingredient troclosene-sodium (sodium dichloroisocyanurate, SDIC) at 2,000 ppm, was efficient in disinfecting CGMMV-contaminated grafting knives in 2 s. Similarly, immersing virus contaminated grafting knives for 2 s in 20% (wt/vol) non-fat milk powder reduced infectivity of the contaminated knives. CGMMV-contaminated nursery sowing trays could constitute a primary infectious viral source transmitted via irrigation water. CGMMV-contaminated sowing trays immersed in KlorBac 2,000 ppm or active oxygen (Huwa-San TR-50) 1%, were efficiently disinfected. Interestingly, hydrophobic insulation of the CGMMV-contaminated trays using dry silicone layers reduced initiation of the viral primary infection in CGMMV-contaminated new sowing trays but was less efficient in CGMMV-contaminated re-used trays. Importantly, Septadine (0.5% chlorhexidine gluconate) was not effective in disinfection of grafting knives. Notably, CGMMV-infected cucumber plant extract incubated with 20% (wt/vol) non-fat milk powder was refractory to the milk suggesting that virus release from surfaces did not necessarily involve virus inactivation.

KEYWORDS

grafting, insulation, MENNO-Florades, silicone, sowing trays, *Tobamovirus*, troclosene-sodium, virus-transmission

1 | INTRODUCTION

Cucumber green mottle mosaic virus (CGMMV), a member of the genus *Tobamovirus*, causes severe disease symptoms in cucurbit cultivars worldwide. The virus, first discovered in 1935 in the United Kingdom (Ainsworth, 1935), spread globally (Dombrovsky, Tran-Nguyen, & Jones, 2017) and recent outbreaks have been reported in Canada (Ling, Li, & Zhang, 2014), United States (Tian et al., 2014), Spain (Celix, Luis-Arteaga, & Rodriguez-Cerezo, 1996; Crespo, Janssen, García, & Ruiz, 2017) and Australia (Tesoriero et al., 2015). In

Israel, the virus was first detected in 1990 (Antignus, Pearlsman, Benyoseph, & Cohen, 1990) and it became a challenge to manage the viral disease because of its outbreak in 2007 in watermelon (Reingold, Lachman, Koren, & Dombrovsky, 2013), cucumber and melon plants (Reingold et al., 2016; Smith & Dombrovsky, 2019; Smith, Luria, Reingold, et al., 2018). Cucurbit cultivars in open fields, greenhouses and nurseries have suffered grave economic losses. Plants infected by the virus generally exhibit severe mosaic, mottling and systemic leaf deformations (Antignus et al., 2001). In watermelons, fruit flesh decay was reported (Reingold et al., 2013; Shim, Lee, Hong, Han, & Kim, 2006).

The tobamoviruses are rod-shaped particles encapsidating positive sense single-stranded RNA (+ssRNA) genomes. The viruses are highly stable with long-term viability on varied surfaces. Members of this group may remain infectious for several months in greenhouse structures, trellising ropes and seed surfaces (Fletcher, 1969). The tobamoviruses could be transmitted by insufficiently treated contaminated seeds and mechanically via planting tools (Broadbent & Fletcher, 1963; Reingold et al., 2016), plant debris (Rao & Varma, 1984), soil (Dornai, Mingelgrin, Frenkel, & Bar-Joseph, 1993; Varveri, Vassilakos, & Bem, 2002) and irrigation water (Vani & Varma, 1993).

In modern agriculture, cucurbits are commonly grafted either on intraspecific rootstocks (Cohen, Burger, Horev, & Koren, 2007) or interspecific rootstocks (Lee & Oda, 2010). Importantly, cucurbit grafting could reduce viral infection from contaminated soil (Cohen et al., 2007; Cohen, Dombrovsky, & Louws, 2017; Edelstein, Cohen, Gur, et al., 2017; Smith et al., 2018). However, grafting knives could mediate viral secondary spread. Similarly, planting/sowing trays, commonly used for seed sowing and for transplanting procedures at the nurseries, may be vehicles for viral primary infections. Furthermore, injured roots of seedlings pulled out of the trays are susceptible to infection by viral inoculum. In an effort to minimise or eliminate plant disease spread, several disinfection methods have been suggested. Among those were ozonization, peroxide treatment (Runia, 1995), iodination, UV irradiation (Runia, 1995) or the use of MENNO-Florades (9% benzoic acid) (Li, Baysal-Gurel, Abdo, Miller, & Ling, 2015) and different types of bleach (Lewandowski, Hayes, & Adkins, 2010; Runia, 1995). However, only a finite number of those treatments were successful in plant production facilities. Furthermore, many disinfection treatments were efficient only for certain viruses (Runia, 1995) and most disinfectants have phytotoxic effects (Xu, Magen, Tarchitzky, & Kafkafi, 1999). The current research focused on finding efficient CGMMV disinfectants that would be applicative for control of CGMMV spread initiated in nurseries.

2 | MATERIALS AND METHODS

2.1 | Chemicals tested for virus inactivation, nursery facility disinfection and sowing tray insulation

The following chemicals were tested at the indicated concentrations in water: MENNO-Florades (9% benzoic acid) (MENNO CHEMIE-VERTRIEB GMBH, Germany), tested at 2 and 4% benzoic acid; Klor Bac, with the active ingredient Troclosene Sodium ($C_3Cl_3N_3NaO_3$) (Concept, Israel) 1,000 ppm, 2,000 ppm; ChloRun (Sodium Dichloroisocyanurate 56% chlorine) (ICL, Israel) 1,000 ppm, 2,000 ppm; Green Up AB, D (Green life group) 10, 20%; Nonfat milk powder (Sigma, Regilait, Casino) 2, 20%; Septadine (Chlorhexidine gluconate) 0.5% (Floris, Israel). Virkon S 2%; Virocid (CID LINES N.V., Belgium) 1 and 3%; Calcium hypochlorite ($Ca(ClO)_2$) 1,000 ppm, 2000 ppm; Active oxygen (silver stabilised hydrogen peroxide, Huwa-San TR-50) 1%; Dry silicone polymer (C.A.S. No.63148-62-9 Poly [dimethylsiloxane], 3 M [TM] Silicone Lubricant, 3 M Center, St. Paul, MN). The various chemical preparations were applied with no addition of spreaders.

2.2 | Double antibody sandwich—enzyme-linked immunosorbent assay

Apical leaf samples were ground in sample buffer containing 2% polyvinylpyrrolidone (MW = 40,000) in Phosphate buffered saline and analysed for CGMMV by Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) (Clark & Adams, 1977) using commercial CGMMV antibodies and alkaline-phosphatase conjugate, each diluted at a ratio of 1:1,000 (Agdia). Substrate (p-nitro phenyl phosphate: Sigma, Steinheim, Germany) was used at a concentration of 0.6 mg/ml. Samples were considered positive when optical density (O.D.) of substrate absorbance values read at 405 nm by an ELISA reader (Anthos, Salzburg, Austria) exceeded three times the negative control reference.

2.3 | Western blot analysis

Western blot was performed as previously described (Dombrovsky, Sapkota, Lachman, Pearlsman, & Antignus, 2013). Apical leaf samples were ground in urea-SDS-β-mercapto-ethanol (USB) buffer, boiled for 10 min followed by centrifugation. The supernatant was mixed with Laemmli loading buffer and proteins were separated on 12% SDS-PAGE (Laemmli, 1970). The gel was subjected to semi-dry electro-blotting (Bio-Rad) for 30 min at 200 mAmp onto a nitrocellulose membrane. The membrane was probed with specific CGMMV antibodies and the results were analysed using alkaline-phosphatase conjugated goat anti-rabbit antibodies. The proteins were visualised by adding the substrate (NBT, BCIP; Promega).

2.4 | CGMMV inactivation assay

Cucumber plants (*Cucumis sativus* cv. Romi) were grown in 0.3-L pots for a week, until maturation of a first true leaf was attained. For inactivation assays, a mixture of virus and the various disinfectants was prepared. Leaves of cucumber plants inoculated with CGMMV-Ahituv isolate (CGMMV-Ah, see GenBank acc. No. KF155232) were ground in 0.01 M phosphate buffer pH = 7.0 (1 g/5 ml) and the suspension was mixed with each disinfectant (1:1, vol/vol), and incubated for three time-periods (1, 5 and 30 min). The mixed suspensions were

subsequently used for a bioassay and were inoculated to 3, 5 or 10 seedlings for each time-period, by rubbing the leaves. Concurrently, a similar number of seedlings were either inoculated with water or CGMMV suspension, and served for negative (un-inoculated) and positive (CGMMV inoculated without disinfectants) controls, respectively. The cucumber seedlings were kept for 16 to 21 days post-inoculation (dpi) in a greenhouse with controlled temperatures (25°C) and 16-h light/8-h dark photoperiods to allow symptom development. Visual inspection was performed and quantification of the virus in newly grown leaves of the treated seedlings was evaluated using DAS-ELISA. The experiment was repeated three times.

2.5 | Viral disinfection of CGMMV-contaminated grafting knives

Cucumber plants (*cv.* Romi) were grown in 0.3-L pots for a week, until a true leaf stage was reached. Plants were inoculated with CGMMV using CGMMV-contaminated grafting knives (razor blades or scalpels) dipped in water or the various disinfectants tested. The knives were infected by cutting a batch of CGMMV-infected cucumber leaves. Subsequently, the knives were dipped in the disinfectant liquids or water, for 2 s. Cucumber plant inoculation was carried out by cutting the lower second leaf. Three to four experiments were conducted with each disinfectant using a minimum of 15 plants and a maximum of 30 plants per experiment. Concomitantly, in five experiments, two sets of five cucumber plants in each, plants were inoculated with a CGMMV-contaminated knife (no treatments) for a positive control. For a negative control, a new knife (sterile) was used. The cucumber plants were kept for 21 days and then inspected for symptoms and virus quantification using DAS-ELISA.

2.6 | Irrigation water contribution to virus spread

Analysis of the contribution of irrigation water to CGMMV spread was conducted by CGMMV inoculation of four plants (cucumber seedlings *cv.* Romi) planted in the middle of a clean planting tray. Six independent experiments were conducted. These experiments were carried out in polyethylene-covered chamber structures as previously described (Frenkel et al., 2016). Commercial nursery trays (66 cm × 33 cm, 171 seedlings in each tray) of cucumber seedlings were grown in a commercial nursery (Hishtil, Israel). Six days after sowing, three trays were positioned in each irrigation chamber. Within each tray four seedlings located in the centre of the nursery tray (i.e., source seedlings) were removed from the tray and were mechanically inoculated with an extract from CGMMV-infected plants that were homogenised in phosphate buffer (0.01 M, pH = 7.0) containing carborundum dust (silicon carbide). Then, the seedlings were repositioned in their original cells. The inoculated seedlings served as the source of infection for the non-infected seedlings growing in the same tray. Care was taken to avoid contact between the inoculated seedlings and the neighbouring healthy ones. The experiments consisted of two irrigation treatments. In the first treatment, the seedlings were irrigated by sprinklers installed above the seedlings at a height of 100 cm. The sprinklers produced droplets ranging in size from 10 to 20 μm. In the second treatment, the seedlings were irrigated by a sub-irrigation system in order to prevent the spread of viral particles by water droplets on the leaves during irrigation. Irrigation was carried out twice daily. Trays with un-infected plants served as negative controls, eliminating possible CGMMV contamination of the water source. Three weeks post CGMMV inoculation plants were analysed by DAS-ELISA. In addition, we examined the contribution of irrigation water to viral disease spread by placing an open plastic vessel over one sap-inoculated cucumber plant (*cv.* Romi) placed in the middle of a planting box, separating the infected plant from adjacent plants. Three independent experiments were conducted. CGMMV spread was monitored a month later using DAS-ELISA.

2.7 | Viral disinfection or insulation of transplanting trays

Nursery new or re-used empty polystyrene 84-cell trays, employed for sowing, rooting, seedling production and plant propagation, were inoculated by spraying CGMMV-infected cucumber plant extract, homogenised in 0.01 M phosphate buffer pH = 7.0, to run-off. The trays were left to dry for 48 h at 25°C, 50% relative humidity and then served for the disinfection experiments. Trays were either immersed in solutions of the various disinfectants for disinfection treatments or were left un-treated, for a positive control. For tray insulation, the CGMMV inoculated trays were sprayed with the silicone polymer either once or four times to create one or four layers of hydrophobic coating, respectively. Fourteen grams of dry silicone polymer were applied on the trays in each layer for uniform coverage of all surface areas of the trays. For the bioassay designed for this experiment, we wounded the cucumber (*cv.* Romi) seedling roots by trimming the root tips (chopping the edge of the root-plug) prior to seedling replanting in the CGMMV-contaminated-treated trays. The root trimming increased the efficiency of infection, which allowed the study of disinfectant efficacy under stringent infection conditions. In the planting procedure, we kept a single empty cell spacer between each seedling, planted in alternating cells within staggered rows, in order to avoid contact during plant growth. Importantly, about 9,000 seedlings were tested in this study, strengthening the validity of the results. The plants on the contaminated trays were grown for 3 weeks allowing CGMMV symptom development and analysis of virus content in the plants was performed by collecting leaf samples for DAS-ELISA.

3 | RESULTS

3.1 | Potential efficient disinfectants for CGMMV inactivation

In order to analyse the efficacy of various disinfectants for CGMMV inactivation, sap from CGMMV inoculated cucumber plants was

incubated with the disinfectants for three time-periods followed by a bioassay on cucumber seedlings for quantification of CGMMV inactivation efficiency. In all bioassays conducted in this work, DAS-ELISA results of infected apical leaves amounted to 0.485–0.953 O.D. value range while the negative control O.D. value range was 0.012–0.031. Infected symptomatic leaves tested by western blot analysis showed a prominent ~15 kDa viral coat protein specific band (Figure 1a,b). The infection ratios obtained in the bioassays, as analysed by DAS-ELISA, were consistently mitigated following incubation of CGMMV-contaminated sap with the reagents: MENNO-Florades 2 and 4%, Virocid 3% and the environmentally friendly product Green Up D 20% (Table 1). The CGMMV-infected cucumber leaves were refractory to nonfat milk 20% or the active oxygen Huwa-San 1% treatment. Importantly, the direct application of CGMMV-disinfectant mixtures on the cucumber leaves often affects leaf morphology. The Green Up D 20% solution treatment occasionally caused necrotic symptoms. Direct application of MENNO-Florades and Huwa-San 1% caused phytotoxicity manifested by bleaching symptoms on the inoculated leaves. Following 3–5 days, emerging new leaves showed no phytotoxic symptoms (Figure 1c–h). Viral disinfection treatments that were efficient only following long incubation periods with the viral preparation were the stabilised chlorine formulation Klor Bac 2,000 ppm, Green Up D 15% and Green Up AB 20%, all prevented viral infection after 5 min of incubation (Table 1).

3.2 | Disinfection of CGMMV-contaminated grafting knives

It has been documented that CGMMV-contaminated knives or shears mediate mechanical transmission of tobamoviruses to at least four plants in non-sequential order (Reingold et al., 2016). We therefore studied several reagents for their capacity to disinfect the grafting knives by short incubation of CGMMV-contaminated knives with various disinfectants, followed by a bioassay on cucumber seedlings for quantification of the disinfection effect. Treatments of CGMMV-contaminated grafting knives with various disinfectants were carried out for 2 s before performing the bioassay on cucumber seedlings (via cuts in two leaves). The obtained results showed that the treatment of the infected knives with tap water resulted in a high infection ratio

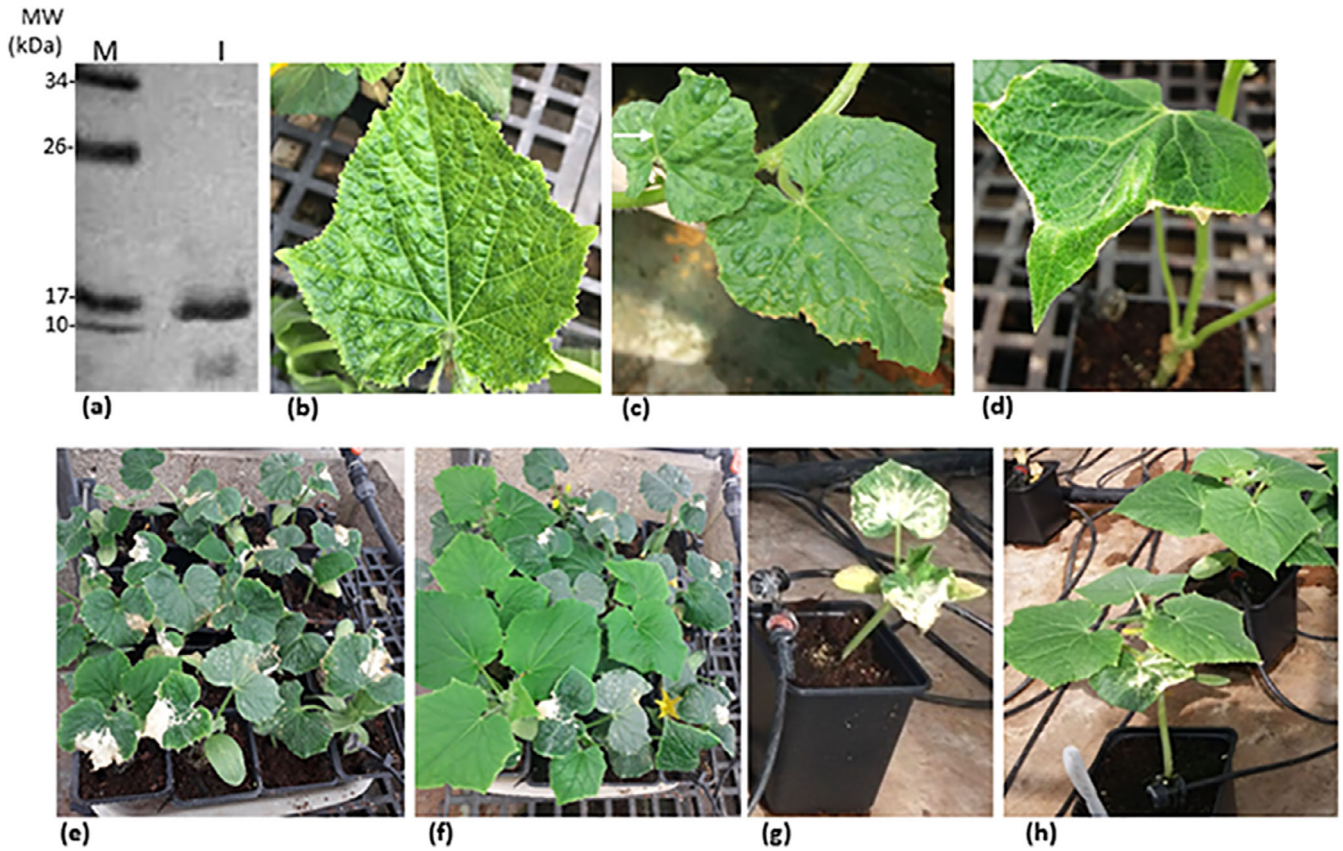

FIGURE 1 Biological experiments for cucumber green mottle mosaic virus (CGMMV) inactivation assays by incubation of disinfectants with virus infected plant extracts. (a) Western blot showing virus coat protein in infected cucumber leaves. (b) A CGMMV-infected cucumber leaf showing mild mottling. (c) Nonfat milk 2 and 20% do not prevent virus sap infection of cucumber leaves. The arrow points to symptoms on the leaf. (d) A cucumber leaf treated with Green Up D 20% disinfected viral extract. (e) MENNO-Florades phytotoxic effect on cucumber leaves. (f) New emerging leaves of MENNO-Florades treated cucumber plants showed no phytotoxic effect. (g) Active oxygen (Huwa-San) 1% solution phytotoxic effect on cucumber leaves. (h) New emerging leaves of (Huwa-San) 1% treated cucumber plants showed no phytotoxic effect. M, protein molecular weight standard; I, CGMMV-infected cucumber leaf extract

TABLE 1 Disinfectant efficiency in inactivation of cucumber green mottle mosaic virus (CGMMV)

	[a]CGMMV infection ratios								
	Exp. 1			Exp. 2			Exp. 3		
Treatment	1 min	5 min	30 min	1 min	5 min	30 min	1 min	5 min	30 min
Water	0/3; 0/3; 0/3; 0/5			0/3; 0/3; 0/3; 0/3			0/3; 0/3; 0/3; 0/3; 0/5		
CGMMV sap	3/3; 3/3; 3/3; 10/10; 6/6			3/3; 3/3; 3/3; 5/5			3/3; 3/3; 3/3;5/5		
MENNO-Florades 4%	**0/10**	**0/10**	**0/10**	-			-		
MENNO-Florades 2%	**0/10**	**0/10**	**0/10**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**
Klor Bac 2,000 ppm	1/3	**0/3**	**0/3**	3/3	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**
Virkon S 2%	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3
Virocid 3%	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**
Virocid 1%	1/3	1/3	0/3	1/3	1/3	0/3	1/3	0/3	0/3
Huwa-San 1%	1/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Nonfat milk 2%	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Nonfat milk 20%	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3
Green Up D 20%	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**
Green Up D 15%	0/3	0/3	0/3	0/5	0/5	0/5	1/10	0/10	0/10
Green Up AB 20%	0/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	0/3

[a]The most effective CGMMV inactivation results were highlighted with bold numbers.

of 75%, eliminating a possible contribution of virus dilution effect that could be attributed to any of the washes with the disinfectants (Figure 2a). Importantly, nonfat milk powder 20% (wt/vol) and Klor Bac 2,000 ppm were the preferred disinfectants for contaminated knives showing a mean of 6 and 7% infection ratios, respectively (Figure 2c, i). The reagents Green Up AB 10% and Green Up D 10% were not as efficient, each showing a mean of 11% infection ratio of the tested seedlings (Figure 2f, g). Between 16 and 22% mean infection ratios were observed when studying the effects of nonfat milk powder 2% (wt/vol), Virocid 1%, active oxygen (Huwa-San) 1% and Virkon 2% (Figure 2b,d,e,h). Importantly, the highest CGMMV mean infection ratio occurred in the biological assay of knives washed with Septadine 0.5%, reaching 32% (Figure 2j). The effect of un-treated CGMMV-contaminated knives, serving as a positive control, amounted to 60% mean infection ratios (Figure 2k).

3.3 | Irrigation water contribute to virus spread in sowing trays

CGMMV-infected sowing trays could occur in nurseries following a previous cycle of sowing of CGMMV-contaminated seeds. We examined the possibility that the infected trays could preserve infectious viral inoculum that would initiate a primary infection in new seedlings. For this scenario to occur the best vehicle for virus transmission would be water. We therefore tested the contribution of irrigation water to CGMMV spread in seedlings grown in sowing trays. We have tested the effect of irrigation operated either by sprinklers watering plants from above, or by sub-irrigation (bathing the root-plants from below). We have found that under both irrigation conditions CGMMV was transmitted to un-infected plants surrounding the four CGMMV-contaminated plants planted in the middle of the trays, as confirmed by ELISA. A summary of six independent experiments (Table 2) showed that mean percent CGMMV infectivity, spread by irrigation water, ranged between 0.6 and 44% using sprinklers and was between 2.3 and 73% under sub-irrigation conditions. The negative controls were negative for CGMMV confirming the water source was devoid of the virus. In addition, experiments studying CGMMV spread via irrigation water, carried out by using an open plastic vessel separating a sap-inoculated plant from adjacent uninfected plants, showed CGMMV infection ratios of 40–70, 60–100 and 40–80% (Figure 3). The negative controls lacking an inoculated plant in the planting box were negative for CGMMV confirming that the water source was devoid of the virus.

3.4 | Preventing disease spread from CGMMV-infected sowing trays

Sowing trays apparently could preserve infectivity potential of virus-infected seeds, which is then transmitted to seedlings and spreads from nurseries to growers. An analysis of the effects of disinfectant solutions on contaminated trays in a large-scale experiment, showed that Klor Bac 2,000 ppm was highly efficient in disinfection of CGMMV-infected new and re-used sowing trays (Table 3). Treatment for 60 s was enough to disinfect the contaminated trays, showing a mean of 0% infectivity. Similarly, active oxygen (Huwa-San) 1% solution was highly efficient in disinfecting the CGMMV-contaminated new and re-used planting trays, showing a mean of 0% infectivity as well (Table 3). CGMMV-contaminated trays were not efficiently

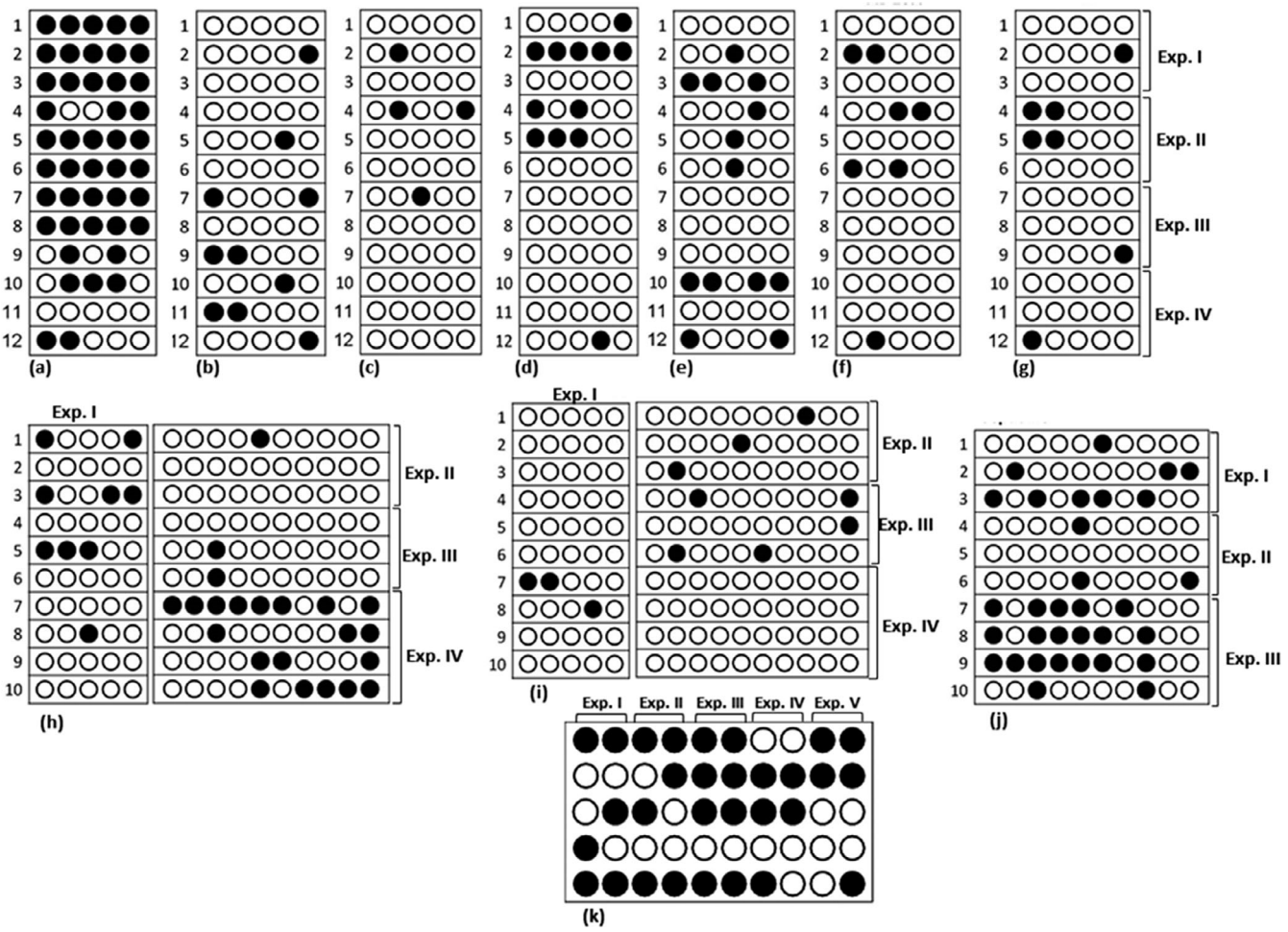

FIGURE 2 Disinfectant efficiency in reducing virus spread via cucumber green mottle mosaic virus (CGMMV) inoculated grafting knives. Virus inoculated knives subjected to a biological assay, carried out for 21 days, after a 2-s dip in various solutions. (a) Water; (b) nonfat milk 2%; (c) nonfat milk 20%; (d) Virocid 1%; (e) active oxygen (Huwa-San) 1%; (f) Green Up AB 10%; (g) Green Up D 10%; (h) Virkon 2%; (i) Klor Bac 2,000 ppm; (j) Septadine 0.5%; (k) Un-treated positive control. Black coloured cells indicate infected plants. White coloured cells indicate un-infected plants

TABLE 2 Irrigation mediated cucumber green mottle mosaic virus (CGMMV) primary infection spread

Irrigation type	No. of exp.	No. of trays	No. of plants	Infection ratios (% ± SD)
Sprinklers	6	16	2,132	17.9 ± 16.6
Sub-irrigation	6	12	1,746	22.5 ± 19.8
Control	6	6	854	0

disinfected by ChloRun unless treatment was conducted for at least 60 s. Nevertheless, the difference between the water-negative control treatment and either ChloRun 1,000 ppm or ChloRun 2,000 ppm, under all experimental conditions, were not statistically significant showing p = 0.205 and p = 0.252, respectively (two sample t-test), suggesting that these chemicals are highly efficient disinfectants. Calcium hypochlorite treatment at 2,000 ppm concentration for 60 s showed 4.8% mean infection ratio in new trays and 14.3% mean infection ratio in re-used trays (Figure 4). Interestingly, hydrophobic insulation of CGMMV-contaminated new trays using dry silicone polymer reduced initiation of CGMMV primary infection in the planted seedlings but the silicone coating was less efficient when applied on virus contaminated re-used trays. (Table 3).

4 | DISCUSSION

Plant viruses differ in their susceptibility to inactivation. Members of the *Potyvirus* genus, for example, Potato virus Y, Tobacco etch virus in tomatoes (Wintermantel, 2011) and Zucchini yellow mosaic virus in zucchini and pumpkin (Coutts, Kehoe, & Jones, 2013) were successfully inactivated by chemical reagents. Unlike potyviruses, members of the *Tobamovirus* genus in general and CGMMV in particular are highly

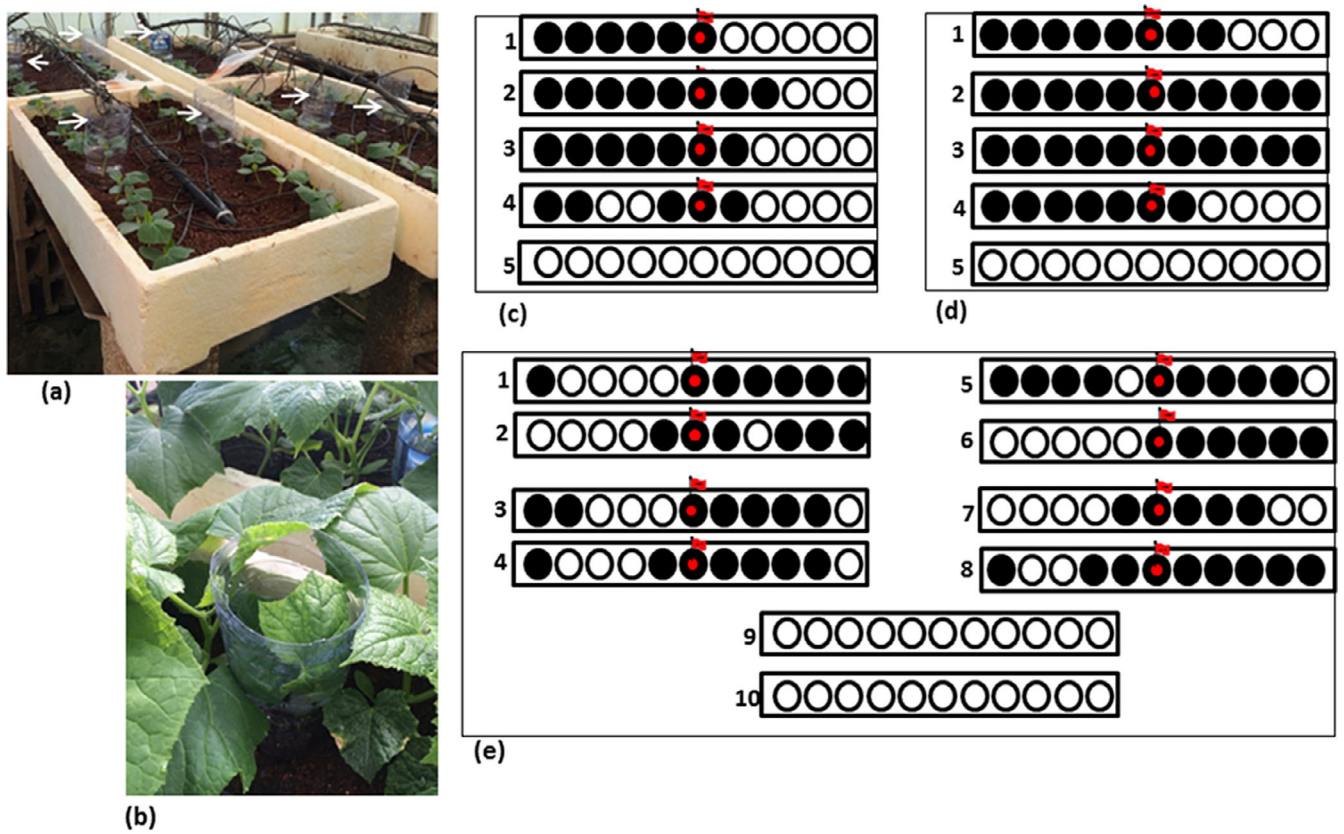

FIGURE 3 Spread of cucumber green mottle mosaic virus (CGMMV) from a primary infection source to neighbouring plants by irrigation water and root-to-root contacts. (a) One CGMMV-infected plant in a planting box separated from adjacent plants by an open plastic vessel placed on the plant. (b) The planting box in (a) 1 month post-inoculation. (c-e) Schemes of planting boxes of three independent experiments depicted in (a and b), 1 month post inoculation. Rows c5, d5, e9 and e10 depict negative controls, having no virus-inoculated plants in the planting boxes. Red flags indicate the inoculated plants. Black coloured cells indicate viral disease spread. White coloured cells indicate un-infected plants. The arrows indicate the isolated inoculated plants

stable and efficiently transmitted mechanically through contact with planting equipment and worker hands (Harper, Hull, Lockhart, & Olszewski, 2002; Rao & Varma, 1984; Reingold et al., 2016). Since its worldwide spread (Dombrovsky et al., 2017; Dombrovsky & Smith, 2017), CGMMV posed a challenge to the discovery of effective disinfectants that would eventually control its high distribution. CGMMV is a seed borne virus and up to date seed disinfection treatments were insufficient for complete inactivation (Reingold, Lachman, Blaosov, & Dombrovsky, 2015). In our current study, we have focused our work on paths of CGMMV spread in commercial nurseries, finding the contribution of sowing trays to primary infections and grafting knives to secondary spread of the viral disease. Indeed, we have shown that CGMMV-contaminated trays could constitute a primary source of disease spread mediated via irrigation water. The CGMMV disease spread efficiently occurred under both modes of irrigation employed: the sprinkler irrigation and sub-irrigation. Sprinkler irrigation increases contact between neighbouring seedlings and injures leaf tissues, we therefore expected the sprinklers to increase infectivity efficiency as occurred in studies of the mechanically transmitted *Clavibacter michiganensis* and *Acidovorax citrulli* (Chalupowicz, Dror, Reuven, Burdman, & Manulis-Sasson, 2015; Frenkel et al., 2016). In our experiments, however, root-to-root disease transmission could also occur, although it was reported to be low (Koh et al., 2018). Our disinfectant efficiency study showed that stabilised chlorine formulation Klor Bac 2,000 ppm was highly efficient in disinfecting grafting knives and sowing trays (Figure 2; Table 3) and less efficient in inactivation of CGMMV as observed in seedling inoculation studies (Table 1). However, 5 min incubation of Klor Bac 2,000 ppm with CGMMV-infected cucumber plant extract did promise successful inactivation of the virus. The most promising reagents in inactivation of CGMMV, tested by seedling inoculation, were MENNO-Florades and the environmental friendly product Green Up-D 20% (Table 1). A lower efficiency in inactivating CGMMV, shown in seedling inoculation studies, was obtained with Virkon S and the efficiency in disinfecting grafting knives was not high as well (Table 1; Figure 2). Unlike our results, Virkon S was reported as an efficient disinfectant for disease spread by the tobamoviruses tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) (Li et al., 2015). Interestingly, active oxygen (Huwa-San) 1% solution was highly efficient in disinfecting sowing trays (Table 3) but was insufficient as a disinfectant of grafting knives and in CGMMV inactivation assays (Figure 2; Table 1). It is possible that adsorption or adhesion of the Huwa-San to tray surfaces

TABLE 3 Disinfectant efficiency in reduction of cucumber green mottle mosaic virus (CGMMV) primary source of infection in sowing trays

	Percent infection ratios (*n* = no. of plants)											
	Exp.1				Exp. 2				Exp. 3			
CGMMV inoculated sowing trays	New trays		Re-used trays		New trays		Re-used trays		New trays		Re-used trays	
Treatment	30″	60″	30″	60″	30″	60″	30″	60″	30″	60″	30″	60″
Klor Bac 1,000 ppm	0.29 (*n* = 336)	0.0 (*n* = 336)	0.0 (*n* = 84)	0.0 (*n* = 84)	14.3 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	1.2 (*n* = 84)	0.0 (*n* = 84)
Klor Bac 2,000 ppm	0.0 (*n* = 252)	0.0 (*n* = 336)	0.0 (*n* = 168)	0.0 (*n* = 168)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)
ChloRun 1,000 ppm	3.2 (*n* = 252)	0.0 (*n* = 252)	1.2 (*n* = 168)	0.0 (*n* = 168)	0.0 (*n* = 168)	0.0 (*n* = 168)	0.6 (*n* = 168)	0.0 (*n* = 168)	-	-	-	-
ChloRun 2,000 ppm	0.0 (*n* = 84)	0.0 (*n* = 84)	1.2 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 168)	0.4 (*n* = 252)	0.0 (*n* = 168)	0.0 (*n* = 168)	-	-	-	-
Huwa-San 1%	-	0.0 (*n* = 168)	-	0.0 (*n* = 168)	-	0.0 (*n* = 168)	-	0.0 (*n* = 168)	-	0.0 (*n* = 168)	-	0.0 (*n* = 168)
Calcium hypochlorite 1,000 ppm	0.0 (*n* = 84)	0.0 (*n* = 84)	57.1 (*n* = 84)	42.8 (*n* = 84)	28.6 (*n* = 84)	0.0 (*n* = 84)	14.3 (*n* = 84)	42.3 (*n* = 84)	57.1 (*n* = 84)	0.0 (*n* = 84)	28.6 (*n* = 84)	0.0 (*n* = 84)
Calcium hypochlorite 2,000 ppm	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)	14.3 (*n* = 84)	14.3 (*n* = 84)	28.6 (*n* = 84)	-	0.0 (*n* = 84)	-	-
Water negative control	0.0 (*n* = 840)											
CGMMV sap positive control	23.2 (*n* = 840)											

	Exp.1		Exp. 2		Exp. 3	
Insulation	New trays	Re-used trays	New trays	Re-used trays	New trays	Re-used trays
Silicone 1-layer	0.0 (*n* = 84)	-	0.0 (*n* = 84)	-	0.0 (*n* = 84)	-
Silicone 4-layers	0.0 (*n* = 84)	1.2 (*n* = 84)	1.2 (*n* = 84)	2.4 (*n* = 84)	0.0 (*n* = 84)	0.0 (*n* = 84)

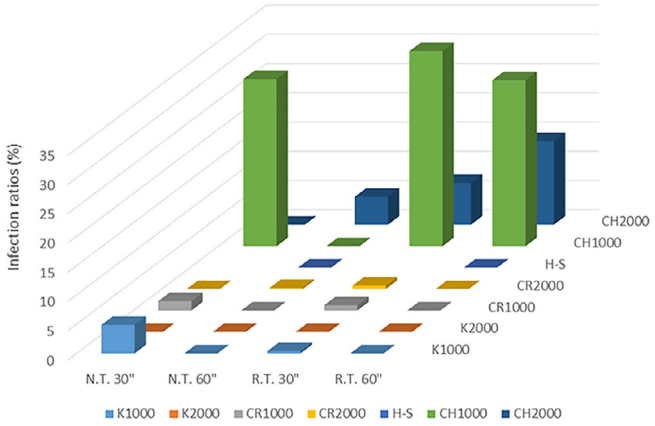

FIGURE 4 Sowing tray disinfection efficiency of various disinfectants. Cucumber green mottle mosaic virus (CGMMV) mean infection ratios in seedlings grown in CGMMV-contaminated new and re-used sowing trays subjected to 30″ and 60″ treatments with four different disinfectants. K1000, Klor Bac 1,000 ppm; K2000, Klor Bac 2,000 ppm; CR1000, ChloRun 1,000 ppm; 2000, ChloRun 2,000 ppm; H-S, Huwa-San 1%; CH1000, Calcium hypochlorite 1,000 ppm; CH2000, Calcium hypochlorite 2,000 ppm. N.T., new trays; R.T., re-used trays

allowed a prolonged period of activity, essential for effective inactivation by oxidizers. Interestingly, insulation of the CGMMV-contaminated new trays, using hydrophobic silicone polymer, reduced CGMMV disease spread (Table 3). Silicone coating, however, was not as efficient in reducing CGMMV disease spread from contaminated re-used trays showing 1.2% infectivity. It is possible that more layers of silicone were necessary to coat scratched or damaged trays. In addition, early hydrophobic protection applied on the trays, prior to the infestation event, with or without additional protective layers, could contribute to improvement of the insulation and reduction of the infectivity potential.

Many disinfection treatments against members of the *Tobamovirus* genus have been tested and published. Unfortunately, suggested solutions to control CGMMV spread within greenhouses and nurseries, failed or were insufficiently effective. Among those were treatments involving the use of chemicals such as Tri sodium phosphate (TSP) for infected seeds, dry heat or both (Kim, Nam, Lee, Yim, & Kim, 2003; Reingold et al., 2015; Sundheim et al., 2008). For decades, the most commonly used disinfectant in agriculture was hypochlorite, which had been used against pathogens on various surfaces, plants and seeds. Sodium hypochlorite, calcium hypochlorite and chlorine bleach were the various products applied in order to achieve maximum disinfection against phyto-pathogens (Gil, Selma, López-Gálvez, & Allende, 2009; Sanz, Giménez, Olarte, Lomas, & Portu, 2002). The household bleach (sodium hypochlorite) was tested for disinfection efficiency of the tobamoviruses hibiscus latent Fort Pierce virus (HLFPV) infecting hibiscus (Kamenova & Adkins, 2004), ToMV infecting tomatoes (Li et al., 2015) and TMV in contaminated razor blades used on petunias (Hayes, 2008). Although previous results showed significant decrease in plant disease incidences, there is still a trend to look for alternative disinfection solutions in order to avoid sodium hypochlorite toxic properties such as suppression of sprout growth, its corrosive effect on surfaces and tools and its loss of effectiveness because of instability (Abdul-Baki, 1974; Reingold et al., 2015).

To circumvent the instability of sodium hypochlorite, several products based on stabilised formulations of chlorine with a relatively low cost were developed. Stabilised chlorine formulations ensured the persistence of the disinfection effect. The calcium hypochlorite used in this study has a neutral pH. The Green UP solutions are completely safe for the user and to the environment and could be used directly on post-harvest fruits and vegetables without leaving any residues. Importantly, direct application of the Green Up solution on cucumber leaves could have secondary harmful effects. Nonfat milk has been used successfully in preventing tobamovirus transmission between plants (Losenge, Faust, & Scott, 2010). In our experiments, nonfat milk powder 20% (wt/vol) showed high efficiency in disinfecting grafting knives, however, the milk was inefficient in inactivation of CGMMV as examined by seedling inoculation assays (Figure 2; Table 1), suggesting that CGMMV inactivation did not occur during the milk treatment of the knives but the virus was washed out of the contaminated knives. To conclude, in order to achieve maximum disinfection of CGMMV we strongly recommend establishing high standard hygienic conduct at the nursery various surfaces using MENNO-Florades 2% solution, active oxygen (Huwa-San) 1% solution and KlorBac 2,000 ppm; these chemicals could be useful as disinfectants of sowing trays as well. Interestingly, coating contaminated new trays with silicone is a promising strategy for tray disinfection.

Agricultural tools, for example, grafting knives could be disinfected using 20% skim milk or KlorBac 2,000 ppm solutions before and during the grafting procedure.

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CONFLICT OF INTEREST

Authors AK, NP and EK are employed by the company Hishtil nurseries Ltd Nehalim, Israel. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

AD, AK, EK, NP Conceptual design of the experiments, ED, OL, AD performed the experiments with disinfectants, ES, AD writing and proof reading of the manuscript, AK supplied the plants, EK, NP performed the planting tray disinfection and the experiments using silicone coating, OF designed and supervised experiments with irrigation water effect, AD designed and supervised the whole project.

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