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RESEARCH ARTICLE

Epidemiological study of *Cucumber green mottle mosaic virus* in greenhouses enables reduction of disease damage in cucurbit production

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Abstract

Since 2007, the tobamovirus *Cucumber green mottle mosaic virus* (CGMMV) has become widespread in Israel, causing severe damage to trellised cucumber and melon in greenhouses and watermelon grown in open fields. To reduce disease damage below the economic threshold, this study focused on four objectives: (a) monitoring the patterns of virus distribution within commercial cucumber greenhouses; (b) studying the potential transmission of CGMMV by agrotechnical activities; (c) virus localization in plant tissues; and (d) searching for techniques that might be adapted for mitigating the disease in trellised cucurbit growth. The results of our surveys demonstrated the role of contaminated seeds and soil as primary inoculum sources, and secondary spread caused by agrotechnical activities. The patterns of secondary disease spread were demonstrated in a series of inoculation experiments involving contaminated knives, shears or hands on wet and dry plants, conducted under research-greenhouse conditions. In parallel experiments using CGMMV-specific antibody and secondary antibody conjugated to Alexa fluor 488, the viral coat protein was visualized in several plant tissues: phloem, xylem, trichomes and grasping tendrils. In addition, commercial-greenhouse experiments were aimed at reducing the number of inoculum sources by identifying and removing infected plants from the plots (early monitoring) prior to agrotechnical activities and/or by adding intermediate medium (IM), such as virus-free compost, to the planting pits at the planting stage. It is suggested that the use of IM combined with early monitoring, awareness of worker mobility (from contaminated structures to young planting areas) and proper sanitation (e.g. disinfection of agrotechnical tools) may reduce the yield losses caused by CGMMV below the economic threshold.

Introduction

Members of the genus *Tobamovirus* are considered remarkably stable because of their unique rigid particles, which encapsidate and protect the viral nucleic acid (Naqvi, 2004). The positive-sense single-stranded RNA tobamoviral genome contains a tRNA-like structure at its 3′ terminus and a methylated-nucleotide cap (m7G5′pppG) at its 5′ terminus, which protect the ends of the RNA strand from degradation (Ono *et al.*, 1972; Ugaki *et al.*, 1991; Tan *et al.*, 2000). Tobamoviruses are known to cause severe crop diseases and are responsible for significant economic losses worldwide in a wide range of plant species (e.g. *Cucurbitaceae* and *Solanaceae*). The symptoms induced by tobamoviruses include mottling and mosaic on leaves, as well as different degrees of fruit mottling or distortion (Fig. 1), which result in severe yield losses in pepper (*Capsicum annum*), tomato (*Solanum*

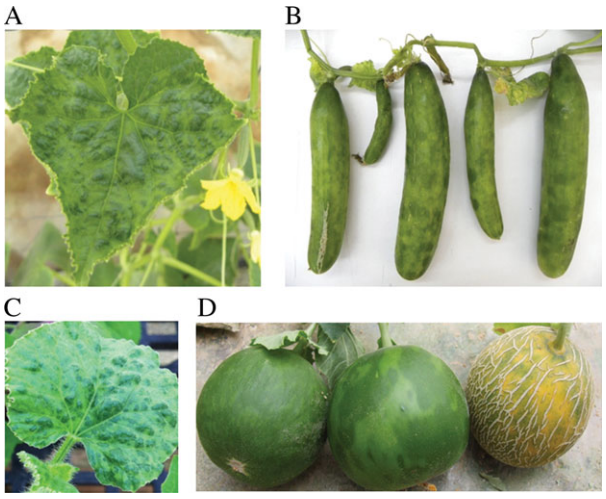

Figure 1 Typical symptoms caused by *Cucumber green mottle mosaic virus* (CGMMV) in cucurbit plants grown in greenhouse conditions. (A and B) Mild green mottling on an apical leaf and fruit of CGMMV-infected cucumber (*Cucumis sativus*) grown in the Ahituv region. (C and D) Mild green mottling on an apical leaf and fruit of CGMMV-infected melon (*Cucumis melo*) grown in the Arava Valley.

lycopersicum), cucumber (*Cucumis sativus*), melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*) (Broadbent, 1976; Wetter *et al.*, 1984; Francki *et al.*, 1986; Ryu *et al.*, 2000; Shim *et al.*, 2005; Reingold *et al.*, 2013).

The tobamovirus *Cucumber green mottle mosaic virus* (CGMMV) was first described in the 1930s as cucumber virus 3 and 4 (Ainsworth, 1935). Since then, the virus has spread among cucurbit crops worldwide, causing damage in the Far East (Ugaki *et al.*, 1991; Yoon *et al.*, 2008), Europe (Celix *et al.*, 1996), India (Sharma *et al.*, 1990) and even Antarctica (Polischuk *et al.*, 2007). The rapid spread of tobamoviruses in general, and CGMMV in particular, without the involvement of an insect vector (Tomlinson, 1987), is mostly because of the stability of their particles, which contributes to their ability to remain infectious under relatively extreme conditions for long periods of time [after several minutes of exposure to solar radiation in space (Orlob & Lorenz, 1968) or within ancient glacial ice (Castello *et al.*, 1999), for instance].

This virus can be transmitted efficiently by mechanical means [e.g. worker hands and tools (Broadbent & Fletcher, 1963; Conti & Lovisolo, 1982; Kamenova & Adkins, 2004)], and remains viable in the soil, enabling its transmission between growth cycles (Dornai *et al.*, 1993). Its viability in and on seeds (Hull, 2002; Agrios, 2005; Reingold *et al.*, 2015) allows it to be easily transferred to distant geographic areas (Taylor *et al.*, 1961; Maule & Wang, 1996; Genda *et al.*, 2011). All these modes of transmission have led to the uncontrollable spread of CGMMV and worldwide damage.

In Israel, CGMMV was first detected in melon plants, where it caused negligible damage (Antignus *et al.*, 1990). Since 2007, outbreaks of CGMMV have occurred in different locations in Israel, including remote areas (e.g. Moshav Na'ama, Dead-sea Valley, coordinates: 31.908327, 35.466513). This epidemic began in cucumber greenhouses in the central part of the country. A year later, the disease could also be found in greenhouse-grown melons and open-field-grown watermelons in the Arava and Jordan valleys, in the south and east of Israel, respectively (Reingold *et al.*, 2013). More recently, in the summer of 2013, the disease was identified in watermelon growing in open fields across the country, causing severe crop damage; the disease caused by CGMMV led to total losses in some growing areas (>1000 ha according to 'KANAT', an Israeli Insurance Fund for Natural Risks in Agriculture). The wide spread of the virus in remote, previously uninfected areas, and in some cases, its appearance in field and greenhouses during the first cycle of cucurbit growth (following a crop that was not a potential carrier of the virus), demonstrated its seed transmission and the importance of seed-disinfection treatments (Reingold *et al.*, 2015). The widespread appearance of CGMMV in the summer of 2013 across the country led us to reinvestigate virus spread and preservation in Israeli production areas.

The main goal of this study was to evaluate and quantify the mode of CGMMV spread via different means of transmission that frequently occur in commercial cucurbit production and to extend the understanding of its viability throughout the production cycles of cucurbit crops. The findings of this study have recently been applied in commercial greenhouses under the framework of the 'CGMMV-Initiative National Project' aimed at reducing the damage caused by the disease below economic thresholds.

Materials and methods

Monitoring the presence and spreading patterns of CGMMV in commercial cucumber greenhouses

Six cucumber greenhouses that had a high incidence of CGMMV disease (>90% infected plants) during the previous growth cycle were selected for the survey. The greenhouses were monitored visually for the presence of CGMMV by walking through the plots at weekly intervals, starting 2–3 days after planting (prior to plant handling, i.e. trellising). Cucumber plants exhibiting typical symptoms of CGMMV infection were labelled and sampled to confirm CGMMV infection by enzyme-linked immunosorbent assay (ELISA) using CGMMV-specific antibodies (Antignus *et al.*, 1990). The

survey was terminated when most of the plants exhibited typical CGMMV symptoms.

CGMMV transmission by hands

Cucumber (*Cucumis sativus* cv. Beit-Alpha) plants (100 plants in 10 repeats) were grown in separate 0.3-L pots for 2 weeks (to the third-real-leaf stage). To inoculate the plants, fingertips were contaminated by touching a CGMMV-infected cucumber leaf once (per repeat) then touching (squeezing) each plant successively in a row (starting with plant no. 1 and continuing to plant no. 10). The cucumber plants were kept for 21 days post-inoculation (dpi) in chambers with controlled temperature (25°C) and a 16-h light/8-h dark photoperiod to allow symptom development. The infection was confirmed by ELISA test.

CGMMV inoculation by knife and shears

Cucumber plants (200 plants in 20 repeats) were grown as described above. Ten repeats were inoculated successively by cutting the stem using shears, and the remaining repeats were inoculated using a knife. Between each repeat, the tools were cleaned with hypochlorite and contaminated by cutting a CGMMV-infected cucumber stem. The cucumber plants were kept for 21 dpi and tested by ELISA as described above. Paired *t*-test ($\alpha = 0.05$) was used to compare the transmission rates of the virus by knife and shears.

CGMMV inoculation of wet versus dry plants

Cucumber plants (100 plants in 10 repeats) were grown as described above. To mimic the guttation effect, five repeats were prewashed with a mist of tap water prior to inoculation, and the others were kept dry. Fingertips were contaminated by touching a CGMMV-infected cucumber leaf once before each repeat. Then the contaminated fingers were passed over the upper parts of the plants within a series in successive order (touching, but not squeezing the leaves). Plants were then kept 21 dpi and tested by ELISA as described above. Paired *t*-test ($\alpha = 0.05$) was used to compare the mean transmission rates on wet versus dry plants.

Transmission of CGMMV through contaminated soil

Soil samples were collected from five contaminated commercial cucumber greenhouses located in Ahituv, Israel (six 10-L pots from each greenhouse; samples collected at a depth of 40 cm using a hoe and shovel). All the soil samples were pooled and mixed. The presence of CGMMV in the homogenised soil was confirmed by transmission electron microscopy and ELISA prior to virion purification. Virion purification from contaminated soil was carried out in six repeats. Briefly, 100 g soil was agitated with 100 mL tap water overnight at 4°C, followed by low-speed centrifugation (10 000 *g*) for 10 min. Virus was then isolated from the supernatant by high-speed centrifugation as previously described for drainage water (Rosner *et al.*, 2006).

Plants were grown in cell trays (Hishtil, Nehalim, Israel) to the second-real-leaf stage. Then the plants were removed from these trays causing root injury, as is typically done under commercial growing conditions and planted in the contaminated soil; 708 plants were planted in 0.3-L pots and grown for 28 dpi and then tested for the presence of CGMMV by ELISA.

Transmission of CGMMV in infected seeds

Five series of 220 CGMMV-contaminated cucumber seeds each [laboratory production (Reingold *et al.*, 2015)] were sown into a clean commercial soil mixture (negative for CGMMV by ELISA) in cell trays. The seeds were grown until the first-real-leaf stage, then pulled from the tray (injuring the root) and immediately returned to the same cell in the tray. The plants were inspected for symptoms at 21 dpi, and symptomatic plants were tested by ELISA to confirm the presence of CGMMV.

In-situ immunofluorescence labelling of CGMMV in infected plant tissues

Healthy (uninfected) and CGMMV-infected cucumber plant tissues (stem, root, leaf, etc.) were dissected with a sterile razor blade and fixed with 4% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde as described previously (Andème-Onzighi *et al.*, 2002; Reingold *et al.*, 2015). The dissected tissues were washed twice using a phosphate-buffered saline (PBS) solution containing 0.05% (w/v) Tween-20 (PBS-T), blocked using PBS with 1% (w/v) milk powder (0% fat) for 30 min and then incubated with specific IgG antibodies against CGMMV (Antignus *et al.*, 1990; Antignus *et al.*, 2001) in the PBS–milk mixture overnight at 4°C. The tissues were washed twice with PBS-T and the secondary antibody, goat anti rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) was added at a 1:200 dilution in PBS, and the tissue samples were incubated for 3h at 37°C with agitation at 100 rpm. The preparations were then washed twice with PBS-T and kept in PBS in a sealed box.

All microscope observations and image acquisitions were performed using the OLYMPUS IX 81- (Shinjuku, Tokyo, Japan) inverted laser scanning confocal

microscope (FLUOVIEW 500) equipped with a 488 nm argon-ion laser. Alexa488 (green channel) was excited by 488 nm light (10–15% power), and the emission was collected through a BA 505–525 filter (Excitation DM 488/543/633, beam Splitter SDM 560). For chlorophyll autofluorescence (red channel), BA 660 IF emission filter was used. The images were colour coded, green for Alexa488 and red for chlorophyll autofluorescence. The transmitted light images were obtained using Nomarski differential interference contrast.

Field implementation of two techniques aimed to minimise the primary inoculum: identification and removal (IR) of sources of primary inoculum versus planting in pits supplemented with intermediate medium (IM) combined with IR

Trellised melon plants were grown in 19 commercial walk-in tunnels in highly CGMMV-contaminated soil (as determined by ELISA). In 10 tunnels (about 16,500 plants in total), the soil was supplemented with CGMMV-free commercial compost (produced from cattle faeces; Shaham, Givat Ada, Israel) as an IM in the planting pits (Fig. S1, Supporting Information). The other nine tunnels (~14 850 plants in total) served as controls with no IM supplementation. In all tunnels, the secondary spread of the disease (through worker activity) was minimised by early IR of infected plants (which might otherwise serve as primary inoculum source) at each time point. CGMMV-infected plants were carefully uprooted, placed in plastic bags and removed from the growing area. Two-way analysis of variance (ANOVA) was conducted using JMP software (SAS Institute Inc., Cary, NC, USA) to detect the statistical significance of IM combined with IR compared with the control treatment (IR only); for this analysis transformed cumulative values (Arcsine) were used at each time point. The Fisher LSD test was used to compare the treatments at each of six time points.

Results

Monitoring of CGMMV incidence in commercial cucumber greenhouses

In 2009, weekly surveys were conducted in commercial cucumber greenhouses in Ahituv, Israel, starting 2–3 days after planting. Leaf samples were collected from first CGMMV-symptomatic plants (first detected ~2–3 weeks post-planting) for assessment by ELISA, and the infected plants were labelled in the greenhouses as sources of primary inoculum because of their early appearance (Fig. 2, marked with red flags and Table 1). The infection of other ~400 randomly selected CGMMV-symptomatic plants, whose symptoms appeared later during the growing season, was confirmed by additional ELISA tests that were conducted towards the end of the survey (5 weeks post-planting). The data obtained from the surveys demonstrated the spreading pattern of CGMMV in trellised cucumber throughout the commercial growth cycle with no intervention. The limited number of primary inoculum sources (3–7 plants per greenhouse; Fig. 2 and Table 1) was sufficient to establish the disease in the greenhouses because of rapid spread of the virus along the rows and to surrounding plants from these sources (Fig. 2 and Table 1). Rapid spread of the disease was observed 1–2 weeks after the performance of intensive agrotechnical work (pruning leaves and trellising, etc.), which involved handling the infected plants and, thus, led to high levels [visual estimation of ≥70% of the plants on average at 65 days post-planting (dpp)] of viral transmission/secondary distribution in the greenhouse via the workers' hands and tools (data not shown).

Transmission rates of CGMMV

To assess the mechanical transmission rates of CGMMV from the inoculum sources to neighbouring plants (secondary distribution/spread) through agrotechnical activities, possible activities were simulated under research-greenhouse conditions. In 10 repeated experiments demonstrating secondary mechanical spread via contaminated fingertips, the infection occurred mostly in a sequential pattern down the row. In 4 of 10 repeats, infection rate was 100% whereas in other, the rate ranged between 50 and 90% with overall 86% infection rate (Fig. 3A and Table 2). The mechanical transmission of CGMMV was not affected by the presence of water on the plants (dry and pre-wetted plants used to mimic humidity and guttation conditions); no statistical differences ($\alpha = 0.05$) were found in the spread of infection on dry as compared with wet plants (Fig. 3B and Fig. 3C and Table 3). Mechanical transmission rate using contaminated tools (shears or knife) was lower than transmission by hands: maximum infection of 5 plants/10 total plants in experiment was observed in one repeat using knife and two repeats using shears; other repeats varied from 1/10 to 4/10 with knife and from 2/10 to 4/10 with shears. No statistical differences were observed between the transmission rates with knife and shears ($\alpha = 0.05$), the average transmission rate was 33%, and no obvious pattern of infection was observed along the rows (i.e. infection was sporadic; Fig. 4A and Fig. 4B and Table 2).

The ability of CGMMV to remain infectious in the soil was evaluated using a mixture of CGMMV-contaminated soils collected from commercial cucumber greenhouses that have had high incidence of CGMMV infection during the previous growing cycle. Healthy seedlings (from CGMMV ELISA-negative seeds) were planted in the

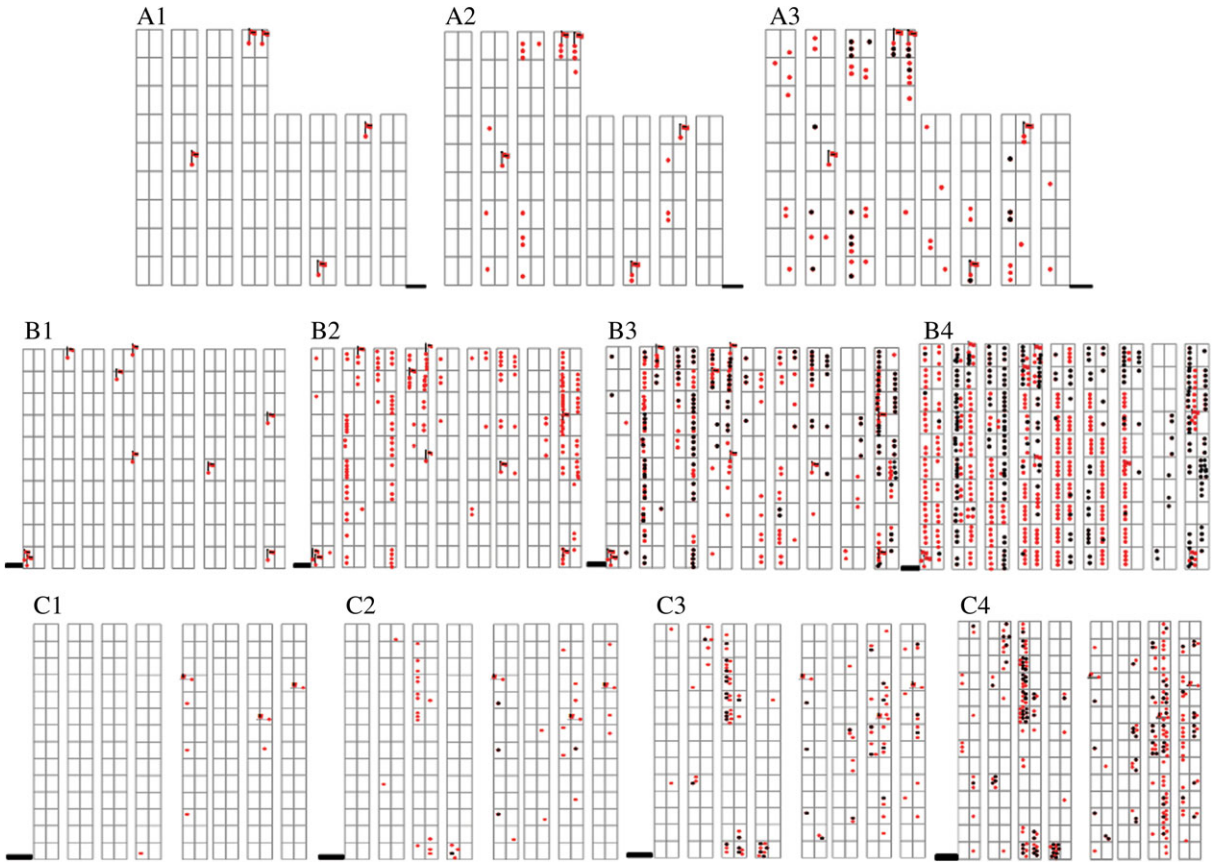

Figure 2 Monitoring the presence and spreading patterns of *Cucumber green mottle mosaic virus* (CGMMV) in commercial cucumber greenhouses in Ahituv. Greenhouse (A) was monitored at three time points, A1–A3, demonstrating the progression of the disease spread up to 21 days post-planting (dpp), 28 dpp and 33 dpp, respectively. The second and third greenhouses surveyed (B and C) demonstrate disease spread at four time points, marked B1–B4 and C1–C4, respectively, up to 21 dpp, 28 dpp, 33 dpp and 40 dpp, respectively. Red flags represent the primary inoculum sources (first identified CGMMV-infected plants); red dots represent infected plants identified after agrotechnical work (e.g. trellising); black dots represent infected plants detected previously (black dots at 33 dpp represents the plants detected at 28 dpp); black rectangles represent the greenhouse entrances. Each greenhouse contained 12–16 rows with 5–15 beds that can hold 10 plants each.

contaminated soil in four replicated experiments. The transmission rate from contaminated soil to plants was 1.13%. Infection in symptomatic plants was confirmed by ELISA (Table 2).

The seed-transmission rates were evaluated by direct sowing of pre-contaminated seeds (extracted from early-infected fruits) into CGMMV-free soil, with five independent repeats. In these experiments, the transmission rate was relatively low: only a single-infected plant was observed (and verified by ELISA) among a total of 1100 symptomless plants (Table 2).

Localization of CGMMV coat protein in cucumber plant tissues by *in-situ* immunofluorescence labelling

CGMMV-coat protein (CP) subunits were detected in the tissues of infected-cucumber plants using CGMMV-specific antibodies (Antignus *et al.*, 1990) and secondary antibodies labelled with Alexa Fluor 488. Strong green fluorescent signals were observed in the xylem vessels and the surrounding phloem sieve tubes in sections of leaf, stem (Fig. 5A–C) and tendril tissues (Fig. 5E). Interestingly, in the leaf sections, clear green fluorescent signals were observed in the trichomes, especially at the tips (marked with arrows in Fig. 5F and Fig. 5G).

Field implementation of two techniques aimed to minimise the primary inoculum sources: IR of primary infected melon plants versus adding IM to planting pits.

The use of IM combined with monitoring and removal of CGMMV inoculum sources to reduce the incidence of disease was evaluated in 10 walk-in tunnels and was compared with a control treatment in which disease was only monitored and sources of inoculum removed (nine walk-in tunnels, IR with no IM supplementation).

Table 1 *Cucumber green mottle mosaic virus* surveys within commercial greenhouses

Green-House Title	Days Post-Planting 14	21	28	33	40	No. of Plants in Greenhouse
	Percentage of Infected Plants					
A	0	0.42	2.08	5.25	11.08	1200
B	0	0.5	9.11	14.83	32	1800
C	0.13	0.36	1.79	4.78	11.21	2240
D	0.08	0.17	1.75	6	13	1200
E	0.52	0.89	3.85	9.56	21.56	1350
F	0.2	0.47	3.07	7.53	17.07	1500
Average	0.16	0.47	3.61	7.99	17.65	
SE	0.08	0.1	1.15	1.54	3.31	

CGMMV infection was first observed in the control structures (0.17%; 25 infected/14 850 total) at 28 dpp. No infection was observed in the IM treatment until 33 dpp, when infection reached 0.05% (8 infected plants of a total of 16 500 plants), whereas in the control, the total infection reached 0.9% (134 infected plants of 14 850 plants) (Fig. 6 and Table 4). In the control structures, the infection rate reached 1.29% (191 infected plants in 14 850 plants in total) at 43 dpp, and no additional infection was detected until 60 dpp (end of the survey), whereas infection rates of 0.11% (16 infected of 16 500 total) and 0.31% (51 infected of 16 500 total) were detected in the IM supplementation at 43 dpp and 60 dpp, respectively (Fig. 6 and Table 4). The two-way ANOVA test conducted on the transformed cumulative values at each time point, demonstrated high statistical significance ($P < 0.001$) for the differences between the treatment (IM combined with IR) and the control (IR), and the differences between the time points (days post-planting). The interaction was significant as well ($P = 0.015$), expressing greater differences between the treatments at later points in time. The Fisher LSD test ($\alpha = 0.05$) was used to compare the treatments at each time point. The results indicated that at early stage of the disease, the effect of IR and IM in combination is more efficient than IR alone (Fig. 6).

Discussion

Since 2007, the widespread of CGMMV has been observed in different parts of Israel, including remote areas, threatening cucurbit crop production (Reingold *et al.*, 2013,

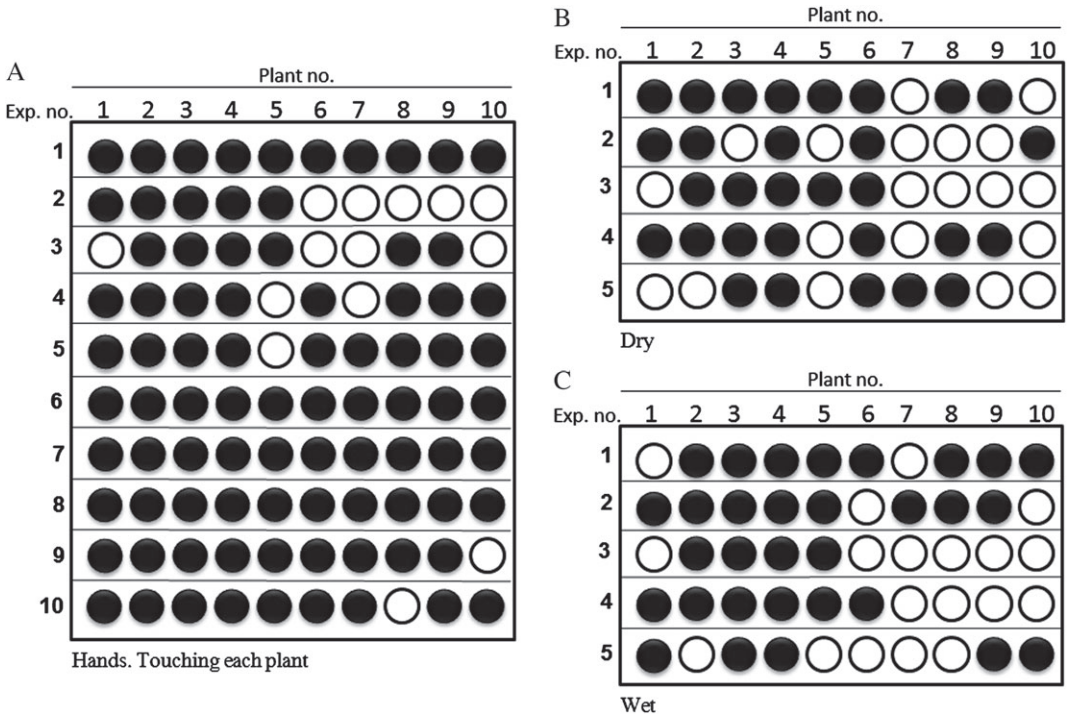

Figure 3 Assessment of mechanical transmission of *Cucumber green mottle mosaic virus* (CGMMV) in cucumber plants under laboratory research-greenhouse conditions. Plants were grown until two-real-leaves-stage and then inoculation was done by (A) squeezing one leaf on each plant with contaminated fingers in successive order (from plant no. 1 to plant no. 10), (B) gentle touch of the upper leaves of dry plants with contaminated fingers and (C) gentle touch of the upper leaves of prewetted plants with contaminated fingers. CGMMV presence was examined by enzyme-linked immunosorbent assay (ELISA) at 21 days post-inoculation.

Table 2 Modes of *Cucumber green mottle mosaic virus* (CGMMV) transmission

Mode of Transmission	Infected Plants	Total Number of Examined Plants	No. Transmission Experiments	Transmission Rate (%)
Hands	86	100	10	86
Tools				
Knife	27	100	10	27
Shears	38	100	10	38
Contaminated soil	8	708	4	1.13
Contaminated seeds	1	1100	5	0.09

Table 3 Transmission experiments of *Cucumber green mottle mosaic virus* (CGMMV) on wet versus dry plants

Mode of Transmission	Infected Plants	Total Number of Examined Plants	No. Transmission Experiments	Transmission Rate (%)
Dry plants	30	50	50	61
Wet plants	31	50	50	62

2015). The virus has been found in trellised cucumber [70% of which are grown in Hefer valley, North Israel (e.g. Ahituv, geographic coordinates: 32.389988, 34.990965)] grown in monoculture with two to three growing cycles a year, trellised melon [up to two growing cycles a year, mostly in the Arava valley, South Israel (e.g. Ein Yahav, geographic coordinates: 30.658260, 35.237851)] and watermelon grown in open fields [one growing cycle per year, across the country (Reingold *et al.*, 2013)]. The widespread CGMMV epidemics combined with the difficulties involved in disinfecting contaminated greenhouses (Broadbent & Fletcher, 1963) and the additional introduction of new CGMMV sources each year via contaminated seeds (Reingold *et al.*, 2013, 2015) have become a major problem for indoor trellised cucurbit crops in Israel. To minimise the disease spread and associated damage, we investigated the pattern of disease spread and assessed selected agrotechniques for disease control that might be applicable on a commercial scale within greenhouse structures.

The data obtained from the surveys carried out in commercial cucumber greenhouses between the years 2009 and 2010 indicated that a large-scale epidemic might start from a few CGMMV-infected plants within a plot (*c.* 0.1% of the plants), identifiable at 14–21 dpp and spread during the growing cycle to the entire plot (Fig. 2). The primary inoculum sources were composed of free virus particles and contaminated plant debris in the soil (Broadbent,

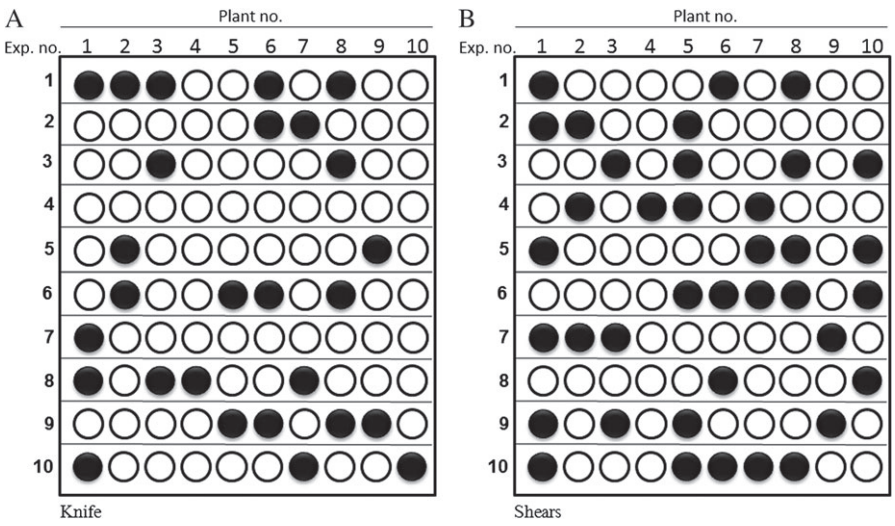

Figure 4 Assessment of mechanical transmission of *Cucumber green mottle mosaic virus* (CGMMV) in cucumber plants under laboratory research-greenhouse conditions. Plants were grown until two-real-leaves-stage and then a contaminated knife (A) or shears (B) were used to infect these plants by cutting in successive order (from plant no. 1 to plant no. 10 in each repeated experiment). CGMMV presence was examined by enzyme-linked immunosorbent assay (ELISA) at 21 days post-inoculation.

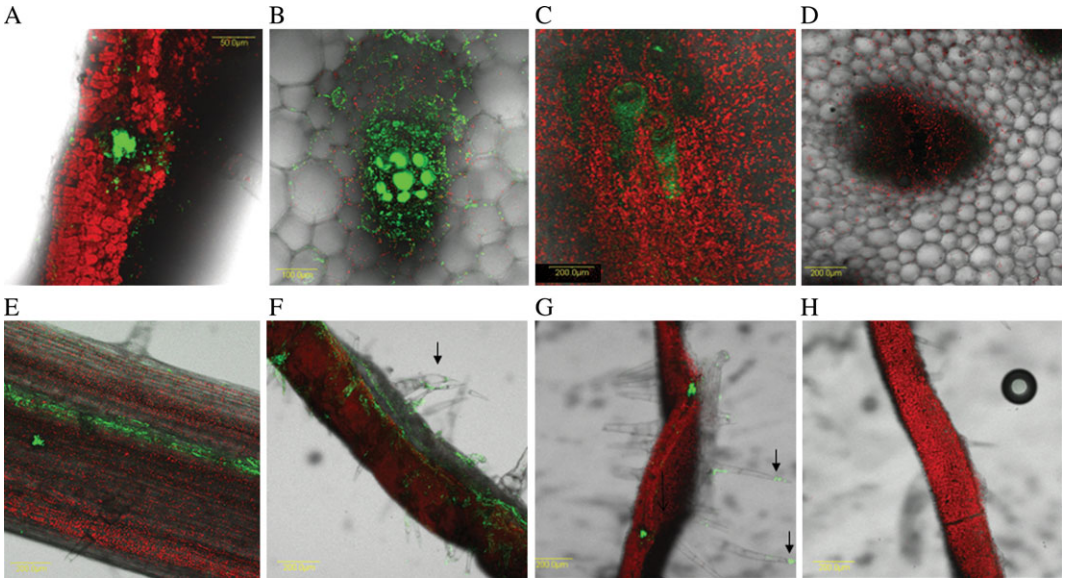

Figure 5 *In-situ* immunofluorescence for localization of *Cucumber green mottle mosaic virus* (CGMMV) in infected-cucumber plant tissues (3 weeks post-planting, 14 days post-inoculation) using CGMMV-specific antibodies and secondary antibody conjugated with Alexa Fluor 488. Green channel indicates the presence of CGMMV coat protein; red channel represents autofluorescence. (A) Transverse section of CGMMV-infected cucumber leaf: green fluorescent signal in plant vessels. (B and C) Transverse sections of CGMMV-infected cucumber stem: green fluorescent signal in xylem and phloem vessels. (D) Negative control using uninfected-cucumber stem. (E) Lengthwise section of CGMMV-infected cucumber grasping tendril: green fluorescent signal observed in xylem vessels. (F and G) Lengthwise section of CGMMV-infected cucumber leaf: green fluorescent signal observed in trichomes (marked with arrows). (H) Negative control using uninfected-cucumber leaf. Bars: (A) 50 μm; (B) 100 μm; (C–H), 200 μm.

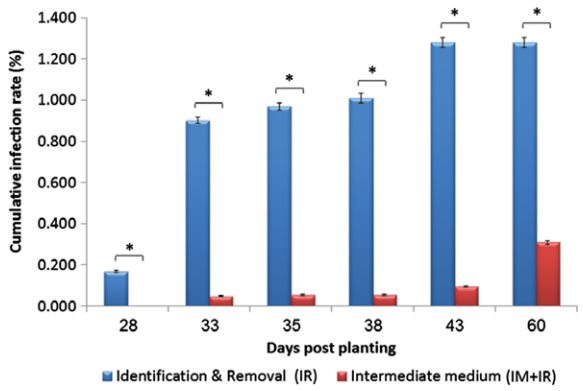

Figure 6 Effect of identification and removal (IR) and intermediate medium (IM) on primary *Cucumber green mottle mosaic virus* (CGMMV) infection in melon plants grown in walk-in tunnels. Asterisks indicate significant differences between the treatments ($\alpha = 0.05$, Fisher LSD test). Bars = standard error.

1965b; Rao & Varma, 1984; Pares & Gunn, 1989; Dornai *et al.*, 1993; Pares *et al.*, 1996; Varveri *et al.*, 2002), and virus carried on or in seeds (Broadbent, 1965b; Moriones & Luis-Arteaga, 1999; Hull, 2002; Agrios, 2005; Reingold *et al.*, 2015). The number of primary-infected plants identified in the surveys (Fig. 2; around 0.4% infected plants) is in accordance with the transmission rates observed by summing the use of CGMMV-infected seeds and planting in CGMMV-contaminated soil (Table 2; around 0. 1% for seeds and 1% for contaminated soil). Similar to other tobamoviruses, the secondary spread of CGMMV occurs mainly via agrotechnical activities performed by workers (Broadbent & Fletcher, 1963; Conti & Lovisolo, 1982; Kamenova & Adkins, 2004). The patterns and efficiency of secondary transmission observed in the surveys at ~21–28 dpp/1–2 weeks after trellising (Fig. 2) are consistent with the results obtained in laboratory experiments (Figs 3 and 4) and with previously reported transmission abilities of other tobamoviruses (Broadbent, 1965a, 1976).

The presence of high levels of viral CP subunits (derived from virus presence) within dissected plant tissues and the delicate structures of the trichomes visualized by confocal microscopy (Fig. 5) can be associated with the high efficiency of the secondary mechanical spread, which occurs because of plant micro-injuries and consequent virus release. Accumulation of the virus within the trichomes can be directly linked to virus transmission by any type of direct contact (even delicate), for example, from touching (hands, work gloves), by tools or trellising ropes (Rao & Varma, 1984). The pattern of virus spread observed in our greenhouse surveys and the laboratory experiments demonstrating spreading ability (Figs 2–4) emphasise the

Table 4 Comparison between two applicable techniques for the reduction of disease damage caused by *Cucumber green mottle virus* (CGMMV) in field experiments

Days Post Planting														
	28		33		35		38		43		60		Total	
Action/ Structure	Infected Plants	Cumulative (%)	Infected Plants	Cumulative (%)	Infected Plants	Cumulative (%)	Infected Plants	Cumulative (%)	Infected Plants	Cumulative (%)	Infected Plants	Cumulative (%)	Plants	Infection Rate (%)*
IR/1	2	0.12	20	1.33	0	1.33	0	1.33	9	1.88	0	1.88	31	1.88
IR/2	8	0.48	18	1.58	4	1.82	0	1.82	3	2.00	0	2.00	33	**2.00**
IR/3	2	0.12	9	0.67	0	0.67	0	0.67	1	0.73	0	0.73	12	**0.73**
IR/4	0	0.00	6	0.36	2	0.48	2	0.61	1	0.67	0	0.67	11	**0.67**
IR/5	1	0.06	9	0.61	0	0.61	0	0.61	0	0.61	0	0.61	10	**0.61**
IR/6	5	0.30	14	1.15	3	1.33	0	1.33	4	1.58	0	1.58	26	**1.58**
IR/7	4	0.24	16	1.21	1	1.27	0	1.27	7	1.70	0	1.70	28	**1.70**
IR/8	2	0.12	6	0.48	0	0.48	0	0.48	2	0.61	0	0.61	10	**0.61**
IR/9	1	0.06	11	0.73	1	0.79	15	1.70	2	1.82	0	1.82	30	**1.82**
Total	25	0.17	109	0.90	11	0.98	17	1.09	29	1.29	0	1.29	191	**1.29**
SE	0.83	0.05	1.71	0.14	0.49	0.16	1.65	0.17	1.00	0.20	0.00	0.20	/	**0.20**
IM/1	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	**0.00**
IM/2	0	0.00	1	0.06	0	0.06	0	0.06	1	0.12	2	0.24	4	**0.24**
IM/3	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	0.06	1	**0.06**
IM/4	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	8	0.48	8	**0.48**
IM/5	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	3	0.18	3	**0.18**
IM/6	0	0.00	1	0.06	0	0.06	0	0.06	1	0.12	2	0.24	4	**0.24**
IM/7	0	0.00	2	0.12	0	0.12	0	0.12	2	0.24	13	1.03	17	**1.03**
IM/8	0	0.00	2	0.12	1	0.18	0	0.18	1	0.24	5	0.55	9	**0.55**
IM/9	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	0.06	1	**0.06**
IM/10	0	0.00	2	0.12	0	0.12	0	0.12	2	0.24	0	0.24	4	**0.24**
Total	0	0.00	8	0.05	1	0.06	0	0.06	7	0.11	35	0.34	51	**0.31**
SE	0.00	0.00	0.31	0.02	0.11	0.02	0.00	0.02	0.28	0.04	1.40	0.10	/	**0.10**

IM, intermediate medium supplementation followed by IR; IR, identification and removal of CGMMV-primary inoculum.
*Bold values indicate $P = 0.05$.

importance of early identification of infected plants which might serve as source of primary inoculum for later virus-transmission to the entire plot.

The transmission rate observed for CGMMV-infected cucumber seeds (1/1,100; ~0.1%; Table 2) was relatively low compared with a recent report of up to 100% infection from seeds (Liu *et al.*, 2014); the latter rate is much higher than our data as well as those of previous publications, demonstrating around 1% of transmission rate (Broadbent, 1976; Rast & Stijger, 1987). The rate of 100% transmission from seeds does not correspond to our knowledge and experience and might perhaps result from contamination. In contrast to seed transmission, the infection rate obtained through the contaminated soil was 10-fold greater than that detected with CGMMV-contaminated seeds, with an infection rate of 1.13% (Table 2), which is lower than the low-scale experiments conducted on *Tomato mosaic virus* and *Tobacco mosaic virus* (Pares *et al.*, 1996; Fillhart *et al.*, 1998). These infected plants, in a trellised greenhouse should be identified and eradicated as early as possible to prevent further spread of the virus (Broadbent & Fletcher, 1963).

The importance of controlling the primary inoculum sources was further demonstrated in large-scale studies conducted in commercial walk-in tunnels, aimed at minimising the number of primary-infected plants within the plots prior to the performance of agrotechnical activities (Fig. 6 and Table 4). In trellised melon plants, early monitoring and removal of the primary inoculum needs to be performed around 25–35 dpp (this time period is referred to as 'the symptomatic time window', when CGMMV symptoms become visible). The 'time window' is identified based on appearance of the earliest reliable symptoms and may vary among different cultivars and growth seasons (Fig. S2A and Fig. S2B). In cucumber commercial growth, this 'time window' appears at around 12–18 dpp (earlier than trellised melon), but in the summer it may occur even earlier because of the intensive growth of the plants, leading to more profound symptom appearance including early wilting of the infected plants. At this time point, the implementation of the 'time window' (including the IR processes) overlaps with the trellising process (which should be carried out only after the removal of virus sources). Any delay in trellising causes severe damage to the plants reflected in massive plant breakage and lower fruit yield, and thus, the 'time window' for cucumber has to be more variable and requires fine tuning.

An additional method for the reduction of primary inoculum derived from other tobamoviruses (i.e. *Cucumber fruit mottle mosaic virus* and *Pepper mild mottle virus*)-contaminated soil, based on IM supplementation (Antignus *et al.*, 2005; Antignus, 2012), was adapted successfully in this study for CGMMV in large-scale experiments (Figs 6, S1 and Table 4). According to our results, a 75% reduction of disease spread was observed when IM was added directly into the planting pits within the CGMMV-contaminated soil. However, this method cannot stand alone and should be combined with early monitoring for maximal efficiency (Figs 6 and S2 and Table 4).

In addition to minimising the primary inoculum sources, the following practices will contribute to reducing the secondary spread: (a) general awareness of worker mobility between structures (for example, walking into young-plant structures before walking into mature-plant structures where the virus is present); (b) the use and frequent changing of disposable gloves, (c) the use of new or disinfected (with 0.1–0.3% hypochlorite) trellising ropes (Nitzany, 1960); (d) frequent laundering of work clothes (Broadbent, 1976); and (e) regular disinfection of tools during work (Fig. S2C–E) and structures (Broadbent & Fletcher, 1963; Broadbent, 1976).

Further research is required for CGMMV management in cucurbits, aiming at understanding its degradation time scale in contaminated soil, trellising ropes, preservation on tools and within plant debris (focusing on roots and stems); the extended knowledge on the host range of CGMMV in the wild plant reservoir should be considered. And, ultimately, searching for an efficient disinfection treatments for contaminated seeds and soil should be further investigated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Application of intermediate medium (IM) to planting pits in soil contaminated with *Cucumber green mottle mosaic virus* (CGMMV) to reduce primary inoculation in commercial melon plots. (A) Compost containers located at the entrance of walk-in tunnel structures ready for application. (B) Planting pits ready for IM application; red arrows point to plant debris from the previous growing cycle. (C) Suggested method for transporting IM within structures.

Figure S2. (A and –B) The symptomatic time window in commercial melon plots: 28 days after planting. (C–E) Reduction of secondary spread via disinfection of agrotechnical tools using 0.1–0.3% hypochlorite (marked with red circle) during the early, middle and late phases of the production cycle.