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# Seed disinfection treatments do not sufficiently eliminate the infectivity of *Cucumber green mottle mosaic virus* (CGMMV) on cucurbit seeds

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Tobamoviruses induce crop diseases that are responsible for significant economic losses around the world. Like other tobamoviruses, *Cucumber green mottle mosaic virus* (CGMMV) forms highly stable particles that can persist for long periods on plant debris, in soil and on seed surfaces. These particles serve as a primary source of infection, infecting seedlings from which the virus can then be mechanically transmitted to other neighbouring plants. Contaminated seeds also provide a route for the movement of the virus between countries and its introduction into new areas. Effective seed disinfection treatments and the use of uncontaminated seed may reduce the global prevalence of this virus. Several treatments based on the use of heat or chemicals have been reported to effectively eliminate CGMMV and other tobamoviruses from seeds. An evaluation of these treatments on highly contaminated seed lots revealed inconsistent results, which encouraged the construction of a more accurate detection method that combines morphological, serological, molecular and biological analyses in one protocol. The detection of viable (infectious) viral particles in seed treated with heat, trisodium phosphate or a combined treatment, indicates that these treatments are insufficient. The serological detection of CGMMV in the inner parts of infected seeds provides a possible explanation for the inconsistent efficacy of these treatments.

*Keywords:* cucumber, melon, seed treatment, tobamovirus, trisodium phosphate

## Introduction

Diseases caused by viruses belonging to the genus *Tobamovirus* result in severe reductions in fruit yield and quality, leading to serious economic losses in important crops such as pepper (*Capsicum annum*; Wetter *et al.*, 1984), tomato (*Solanum lycopersicum*; Broadbent, 1976) and cucurbits, particularly cucumber (*Cucumis sativus*), melon (*C. melo*) and watermelon (*Citrullus lanatus*; Francki *et al.*, 1986; Ryu *et al.*, 2000; Shim *et al.*, 2005). Tobamoviruses are characterized by their unique rod-shaped viral particles, *c.* 300 nm in length, that include a single positive-sense strand of RNA of *c.* 6·4 kb (Ono *et al.*, 1972; Ugaki *et al.*, 1991; Tan *et al.*, 2000) that encodes replication proteins (126 and 180 kDa), a movement protein (MP, 30 kDa) and a coat protein (CP, 17 kDa) (Dorokhov Yu *et al.*, 1994). Tobamovirus particles are known to be highly stable and can persist for long periods without a living host, in plant debris or by adsorbing to a solid-phase clay fraction in the soil (Lanter *et al.*, 1982; Dornai *et al.*, 1993; Hull, 2002; Varveri *et al.*, 2002). Although tobamoviruses are not known to be transmitted by any insect vector, they are efficiently transmitted by mechanical means such as workers' hands, clothing and tools (Broadbent & Fletcher, 1963). Moreover, tobamoviruses are the only viruses known to be able to survive in an infective state on the surface of seeds, without invading the inner tissues, for several months to a few years (Orlob & Lorenz, 1968; Hull, 2002; Agrios, 2004).

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 (Vasudeva & Singh, 1949; Antignus *et al.*, 1990; Alshahwan & Abdalla, 1992; Odintsova *et al.*, 2000; Polischuk *et al.*, 2007; Chen *et al.*, 2009). The disease symptoms caused by CGMMV include mottling and mosaic symptoms on leaves and different degrees of fruit mottling or distortion.

In cases of widespread epidemics in cucumber crops, total yield losses of up to 15% can be observed, especially when an early infection occurs in a nursery. However, when the infection is delayed for 6 weeks, the yield loss may be less significant (Fletcher, 1969). The supply of primary inoculum may be reduced through the use of seed disinfection methods, as reported in the literature. Some of these methods are based on heat treatment at various temperatures for different periods of time (Kim *et al.*, 2003). Other methods involve the use of acidic or alkaline substances, e.g. 10% trisodium phosphate (TSP; Rast & Stijger, 1987; Cordoba-Selles *et al.*, 2007).

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Plant Pathology

Since 2007, there have been outbreaks of CGMMV in different locations in Israel, including in remote areas. The 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 watermelons and in open fields in the Arava and Jordan valleys (southern and eastern parts of Israel, respectively; Reingold *et al.*, 2013). Interestingly, the disease was also identified in commercial greenhouses of growers on their first cycle of growing cucurbits, which points to seed transmission. In the efforts to identify the source of the primary inoculum, it was noted that cucurbit seed lots that had been treated with chemicals or heat were infected with the virus and served as a source of primary inoculum. The goals of the current study were: (i) to examine the efficacy of disinfection treatments that are widely used on a commercial scale; and (ii) to develop an extensive protocol for the diagnosis of CGMMV in/on cucurbit seeds, to reduce the incidence and spread of the disease to below the economic threshold.

## Materials and methods

### Preparation of infected seeds

*Cucumber seeds*

Two-week-old nonparthenocarpic cucumber *C. sativus* 'Derben' plants were mechanically inoculated with extract from CGMMV-infected plants (Ah isolate, isolated from cucumber, GenBank accession no. KF155232) that were homogenized in 0·01 M phosphate buffer (pH 7·0; PB7) containing carborundum (silicon carbide). The inoculated plants were planted in 10-L pots and placed in a greenhouse covered with 50-mesh netting to continue growing until the appearance of ripe fruits. Pollination was carried out by bumblebees (*Bombus terrestris*; Bio Bee). Three weeks after mechanical inoculation, leaf samples (three upper leaves) were collected from each plant and screened for the presence of CGMMV using CGMMV-specific antibodies (Antignus *et al.*, 1990) in an enzyme-linked immunosorbent assay (ELISA; Clark & Adams, 1977). Seeds were collected from ripe fruits and the presence of CGMMV in those seeds was determined by an additional ELISA.

*Melon seeds*

Ripe melons, *C. melo* 'Raanan', exhibiting typical CGMMV symptoms were collected from plants in commercial greenhouses. The infection of each melon fruit was confirmed by ELISA. Seeds were collected from the infected fruits and further tested for the presence of CGMMV using ELISA.

The infected seeds from both melons and cucumbers were washed extensively with tap water, incubated overnight (16 h) in water while being slowly agitated, and then dried on sheets of paper at room temperature for 24 h before being used in further experiments.

### Purification of the virus from infected seeds

For each seed analysis, 40 g of seeds (*c.* 1100–1300 melon seeds and *c.* 1400–1600 cucumber seeds) were soaked overnight in 50 mL 0·1 M PB7 with low agitation at 4°C. An additional equivalent amount of 0·1 M PB7 was added to the swollen seeds as they were ground in a homogenizer. The homogenized seed extract was first centrifuged at low speed (10 410 ***g***) for 10 min and the supernatant was then collected and subjected to high-speed centrifugation (35 280 ***g***) for 3 h. The obtained pellet was dissolved in 1 mL 0·01 M PB7. The presence of viral particles in the purified material was confirmed using transmission electron microscopy (TEM; Tecnai G2, FEI-Philips), by incubating 5 μL of the purified sample on a copper grid for 5 min, followed by uranyl acetate staining for an additional 5 min. Micrographs were taken using a MegaView III camera operated using ANALYSIS software (Soft Imaging System).

### Viral RNA extraction

*Extraction of viral RNA from purified viral particles*

Viral RNA was extracted from purified virus preparations as described previously (Rosner *et al.*, 1983; Dombrovsky *et al.*, 2012). Purified virus preparations were incubated with RQ RNase-free DNase I (Promega) for 1 h at 37°C. This incubation was followed by proteinase K treatment for 1 h at 37°C. Then, the viral nucleic acid was purified by extraction with a water-saturated acid–phenol mixture (Biological Industries). The aqueous phase of the preparation was precipitated overnight at −20°C in the presence of glycogen (Fermentas-Thermo Fisher Scientific), 0·1 M sodium acetate and isopropanol. The precipitated viral RNA was washed with 75% ethanol and allowed to air dry for 10 min. The dried viral RNA was then suspended in 40 μL 0·01 M Tris-borate EDTA (TBE) buffer.

*Direct extraction of viral RNA from infected seeds*

One hundred CGMMV-infected melon seeds (3·2–3·6 g) were soaked overnight in 10 mL 0·1 M PB7 and then ground. The homogenate was collected and subjected to low-speed centrifugation at 5000 ***g*** for 5 min. RNA was extracted from the clarified solution, as described above (i.e. DNase I digestion followed by proteinase K treatment) and viral RNA was then extracted using a commercial kit according to the manufacturer's instructions (Viral RNA Extraction Kit; Bioneer).

### Characterization and detection of CGMMV RNA

Ten microlitres of suspended RNA (1–5 μg on average) purified from virions was separated by electrophoresis on a 1% TBE agarose gel (100 V for 45 min). In parallel, 5 μg of the purified viral RNA or RNA extracted directly from 100 seeds served as a template for a reverse transcription (RT) reaction carried out using the RevertAid Premium reverse transcriptase enzyme (Fermentas-Thermo Fisher Scientific) and the complementary primer R-*Xho*I designed for the CGMMV 3′ UTR (5′-AAACTCGAGTGGGCCCCTACCCGGGGAA-3′; at position 6424 nt).

First, the obtained cDNA was amplified in a PCR procedure using JMR PCR mix (JMR Holdings) and specific primers flanking the coat and/or movement protein genes [F-CP (5′-GATGGCTTACAATCCGATCAC-3′; at position 5762 nt) and R-CP (5′-CCCTCGAAACTAAGCTTTCG-3′; at position 6248 nt) or F-MP (5′-CATAGATGTCTCTAAGTAAGGTG-3′; at position 4994 nt) and R-CP, respectively]. Then, an additional PCR amplification was performed to verify the completeness of the viral cDNA synthesized using the primer pair F-*Nco*I-5′ (5′-CCTGCCATGGGTTTTAATTTTTATAATTAAACAAACAACA-3′; at position 1 nt) and R-*Sal*I (5′-AATAGTCGACCCCGGGGTTCACATAACATTA-3′; at position 2623 nt), in order to amplify the region from 5′ UTR to nt 2624. In addition, viral cDNA was used for long-distance (LD) PCR using the Advan-

tage 2 Polymerase Mix (Clontech-Takara) and two specific primers designed on the UTRs at both ends of the CGMMV genome (F- *Nco*I-5′ and R- *Xho*I-3′). For characterization of the different PCR products, the obtained mixtures were separated by electrophoresis in a 1% agarose gel (100 V for 30 min) at room temperature.

### Inoculation of test plants with purified virions or RNA extracted directly from seeds

Purified virions (40 g batches of seed) and RNA extracted from seeds (batches of 100 seeds each) were used to inoculate cucumber 'Beit-Alpha', melon 'Arava' and *Nicotiana benthamiana* plants. Inoculum consisted of 10 μL of purified virions taken from a diluted solution of 20 μL virions in 70 μL TBE, or 5 μL of viral RNA extract taken from a solution of 10 μL RNA in 40 μL TBE. These were gently rubbed on each plant dusted with carborundum using a pipette with sterile tips (three or four plants of each of cucumber, melon and *N. benthamiana*). The leaves were carefully washed with tap water (mist) 5 min later. The status of the infected plants was confirmed by the appearance of typical CGMMV symptoms and validated by ELISA 14 days post-inoculation (dpi).

### *In situ* immunofluorescence labelling of CGMMV in seed tissues

The seed coats (testae) of CGMMV-infected melon seeds were separated from the inner seed tissues and each tissue section was sliced and fixed with 4% formaldehyde and 0·2% glutaraldehyde, as described previously (Andeme-Onzighi *et al.*, 2002). The slices were washed twice using a phosphate-buffered saline (PBS) solution containing 0·05% Tween-20 (PBS-T), blocked using PBS with 1% milk (0% fat) for 30 min and then incubated with specific IgG antibodies for CGMMV (Antignus *et al.*, 1990, 2001) in the PBS–milk mixture overnight at 4°C. The slices were washed twice with PBS-T and the secondary antibody, goat anti-rabbit IgG-conjugated Alexa Fluor 488 (Invitrogen), was added to the slices in a 1:200 dilution in PBS and the material was incubated for an additional 3 h at 37°C with agitation. Then, the slices were washed twice with PBS-T and kept in PBS in a sealed box. Seeds from ELISA-negative melons that had been treated with 10% TSP and heat (72°C for 72 h) served as a negative control. The fluorescence signal of the slices was detected using confocal microscopy.

### Verification of the effectiveness of seed disinfection treatments

Three disinfection treatments (10% TSP, heat and a combination of TSP and heat) were evaluated on batches of 40 g of CGMMV-infected melon seeds or on batches of 100 CGMMV-infected melon seeds (3·2–3·6 g). In preliminary experiments, the TSP treatment was applied for 30, 60 and 90 min with slow agitation, followed by an extensive wash with tap water. However, because no significant differences were detected in the ELISA and the bioassay results of these different treatments (data not shown), in later experiments, only the 30-min treatment was applied. For the heat treatment, the seeds were incubated at 72°C for 72 h. In preliminary work, this procedure was carried out at different temperatures and over a longer period. Once the optimal temperature for seed viability was determined, that temperature was used in all further studies. The combined procedure started with the TSP treatment, which was followed by the heat treatment. Molecular (RNA separation on TBE gel, RT-PCR amplification), serological (ELISA), microscopy (TEM), and biological (mechanical inoculation of susceptible plants) analyses were used to evaluate the efficacy of each disinfection treatment on both scales (40 g batches and 100-seed batches; Figs 1 & S1).

Viral purification was carried out on batches of 40 g of untreated or treated seeds (Fig. 1a, left path) and particle morphology was visualized using TEM (Fig. 1b). Then, viral RNA was extracted from purified particles and subjected to RT-PCR amplification (Fig. 1c1–c2). In parallel, the same RT-PCR procedures were performed on batches of 100 seeds (Fig. 1a, right path). In addition, cucumber, melon and *N. benthamiana* plants were mechanically inoculated with purified virions obtained from the 40 g batches or the RNA extracted from the batches of 100 seeds. Twenty seeds in five replicates (100 seeds in total: seven infected treated, eight infected untreated and five CGMMV-free per replicate) were subjected to *in situ* immunofluorescence labelling.

## Results

### Detection of CGMMV in infected seeds

Cucumber and melon seeds were collected from CGMMV ELISA-positive fruits. Presence of CGMMV-CP in the collected seeds was confirmed by further ELISA procedures performed on 20 subsamples of 100 cucumber or melon seeds (a total of 2000 cucumber and 2000 melon seeds). Typical tobamovirus particles with rod-shaped morphology (*c.* 300 nm in length) were observed in TEM analyses of samples of purified virions from 40 g batches of CGMMV-infected cucumber seeds and 40 g batches of CGMMV-infected melon seeds (Fig. 2a). Separation of the viral RNA extracted from purified virions on TBE agarose gel revealed the presence of a viral RNA fraction with an estimated size of *c.* 6 kb (Fig. 2b1). Further detection of CGMMV in seed samples was carried out by RT-PCR amplification of the genome fragment encoding the CP and MP genes. Later, the integrity of the viral genome was analysed by amplifying the entire genome, yielding an amplicon with estimated size of 6 kb (Fig. 2b2). The authenticity of the amplicons was partially verified by nucleotide sequencing of the regions encoding the CP and MP genes.

Three to four plants of cucumber, melon (5-leaf stage), and *N. benthamiana* (3-leaf stage) were also mechanically inoculated with purified virions and extracted viral RNA. Typical systemic symptoms were observed in these plants at 7–9 dpi (Fig. 2c) and the presence of the virus was verified by ELISA.

### Detection and localization of CGMMV coat protein in seed tissues by *in situ* immunofluorescence labelling

The presence of CGMMV CP subunits in infected melon seeds was detected using specific CGMMV IgG antibodies (Antignus *et al.*, 1990) and fluorescently labelled secondary antibodies (goat anti-rabbit) labelled

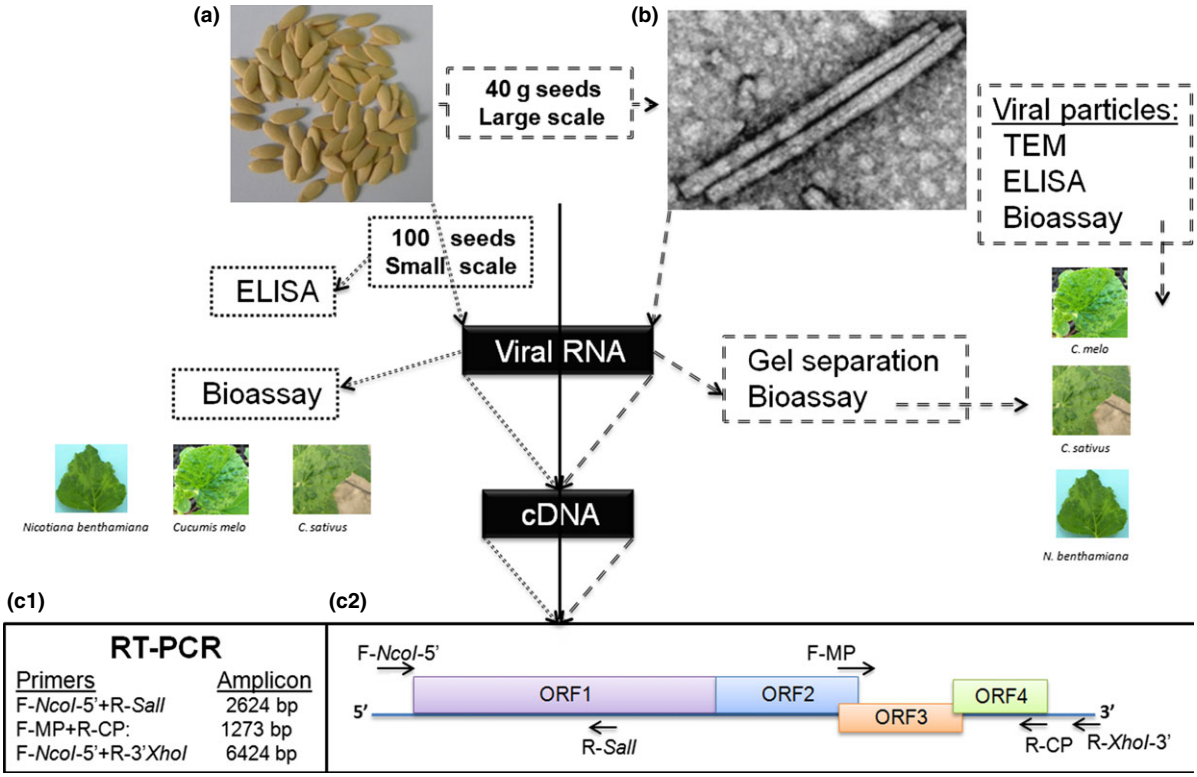


Figure 1 Schematic representation illustrating the two protocols and the detection methods used in this study. Those methods included morphological, serological, molecular and biological analyses in two combined protocols for the detection of CGMMV virions, CGMMV CP or CGMMV RNA in seed lots. (a) Large text boxes and arrows represent the large-scale protocol, in which 40-g batches of seed were used. Small text boxes and arrows represent the small-scale protocol conducted using batches of 100 seeds each. (b) Transmission electron microscopy (TEM) image of CGMMV particle morphology. Viral RNA was detected using gel electrophoresis and a bioassay. Viral particles were detected using TEM, ELISA and a bioassay. The bioassay was based on the mechanical inoculation of three selected susceptible plants (*Nicotiana benthamiana*, *Cucumis sativus* and *C. melo*) with viral RNA or particles. (c1–c2) Reverse transcription (RT)-PCR amplification of genome fragments or the entire genome; (c1) primer pairs and amplicon sizes; (c2) primer binding sites on the viral genome.

with Alexa Fluor 488. Confocal microscopy revealed high fluorescence signals in the testa and endopleura samples from the majority of examined seeds (18/20 seeds; Fig. 3). The seeds that tested positive for the presence of CP subunits were further examined for the presence of CGMMV CP within their inner tissues (i.e. endopleura, embryo). Higher fluorescence signals were noted in the seed coats (testa and endopleura, the outer and inner side, respectively), compared to the inner tissues (endosperm; Fig. 3b,d). However, significant differences in fluorescence signals were identified in the inner tissue of infected versus uninfected seeds (Fig. 3c,d). The signal in the inner tissues was located in the endosperm and surrounding layers, but no fluorescence signal was detected in the embryo/cotyledons of any of the examined seeds.

### Efficacy of seed disinfection treatments

The efficacy of two commonly used commercial seed treatments (Taylor *et al.*, 1961; Rast & Stijger, 1987; Kim *et al.*, 2003) and a combination of those treatments were examined. Specifically, the ability of each treatment to eliminate and inactivate CGMMV in highly infected melon and cucumber seeds was evaluated.

Seed treatments were performed on batches of 40 g of infected seed and batches of 100 infected seeds (large- and small-scale, respectively; Figs. 1 & S1). Each of the 40 g seed samples was analysed using five methods: ELISA, TEM, RNA extraction and separation, RT-PCR (for CP and for the complete viral genome) and a biological assay. A similar approach was used to examine the batches of 100 seeds; however, the TEM and RNA separation were not used for the analysis of those batches.

In total, 24 large-scale experiments were performed on 40 g batches of CGMMV-infected cucurbit seeds This consisted of eight experiments involving 10% TSP treatment, eight involving heat treatments and eight involving the combined treatment. Half of the experiments (four of each treatment) were conducted on melon seeds and half on cucumber seeds. All 24 experiments yielded positive serological results, as detected by ELISA, in both cucumber and melon seeds. Therefore, in order to improve the accuracy and fidelity of the diagnostic methods, it was decided to focus only on the CGMMV-infected melon seeds in further experiments.

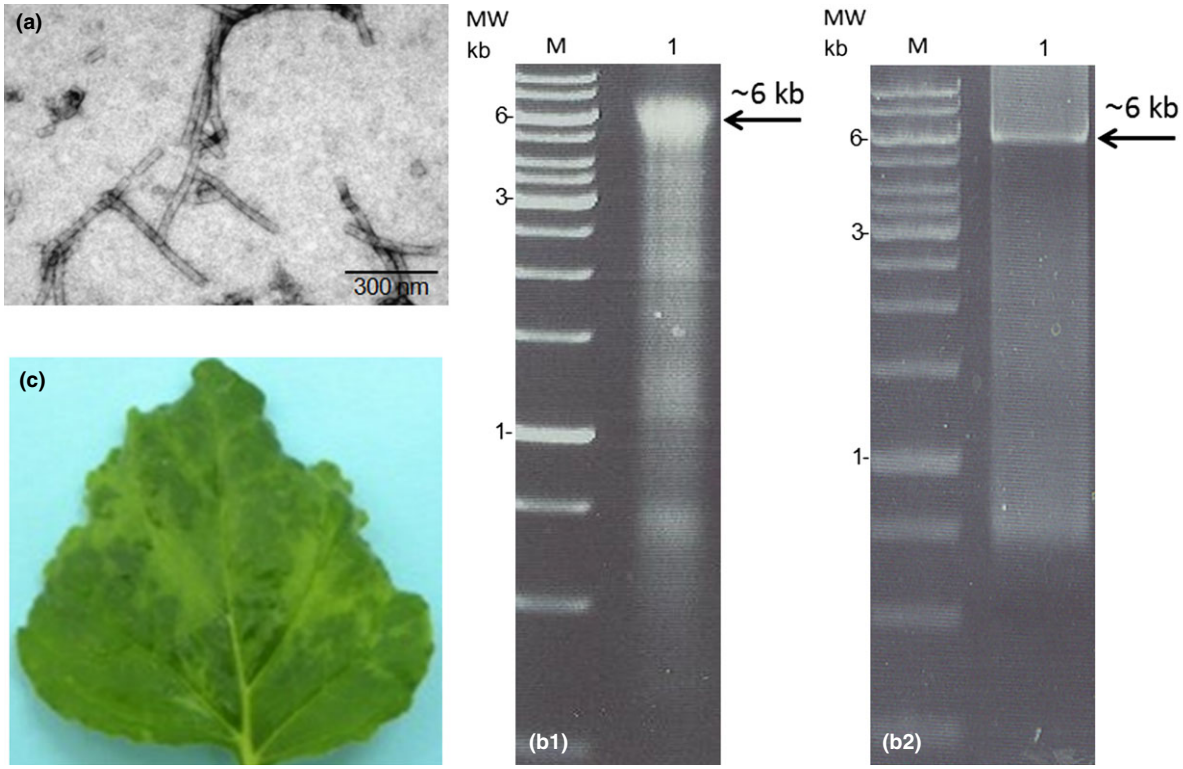


Figure 2 Characterization of CGMMV purified from seeds. (a) TEM image of viral particles purified from 40 g of infected melon seed. (b1) Viral RNA extracted from 40 g of infected seed and separated on a TBE gel (1 $\mu$L out of 40 $\mu$L suspended RNA). The marked band is the full-length viral RNA. (b2) One of the five RT-PCR amplicons representing the entire CGMMV genome. (c) *Nicotiana benthamiana* leaf inoculated with 10 $\mu$L of the 40 $\mu$L of suspended RNA extracted from 40 g of infected seed.

### Particle morphology

Virion purification was performed using 40 g batches of treated seed (TSP, heat and combined treatments). TEM revealed particles with the typical CGMMV morphology (300 nm in length) in all of the virion samples purified from seeds that had undergone the different disinfection treatments (Fig. 4a; summarized in Table 1).

### Viral RNA

RNA of the typical size of tobamovirus genomes was detected in all of the 40 g batches of untreated CGMMV-infected seeds; viral RNA corresponding to a *c.* 6·4 kb band was visible in the TBE gel separation (Fig. 2b1). The entire viral genome was detected in all of the seeds that had been treated with 10% TSP and in the heat-treated seeds. In contrast, this RNA was not detected in seed that had undergone the combined treatment (summarized in Table 1).

### Genome amplification

For seed treatments performed on 40 g batches of infected seeds, RT-PCR was successfully used to amplify genome fragments of the CP (496 bp; Fig. 4b) gene, CP-MP (1273 bp) genes and the entire genome (6424 bp). Moreover, positive amplification of the 5′ region downstream of position 2623 was also detected. The entire genome could not be amplified from seeds subjected to the combined treatment (0/4 repeats), although CP and MP could be amplified from those seeds (Table 1).

RT-PCR analysis of 100-seed batches of untreated seeds (control) allowed the amplification of the CP alone, the CP-MP genes and the entire genome. In the batches of 100 TSP-treated seeds, the CP-MP genes were detected in four out of four experiments, but the entire genome was amplified in only two of four experiments. Dissimilar results were attained from the four replicates of the heat treatment: in all four experiments, CP-MP genes were amplified, but the entire viral genome could not be amplified (0/4; Table 1).

### Biological assay

Susceptible test plants were mechanically inoculated with virions (extracted from 40 g seed batches) or viral RNA (extracted from 100-seed batches) and grown until symptoms developed (10–14 dpi). This system served as a biological assay to complement and validate the laboratory analysis.

The examination of the infectivity of virions extracted from 40 g batches of untreated or treated CGMMV-infected seeds revealed high percentages of infectivity in all four replicates of the positive control (untreated; 4

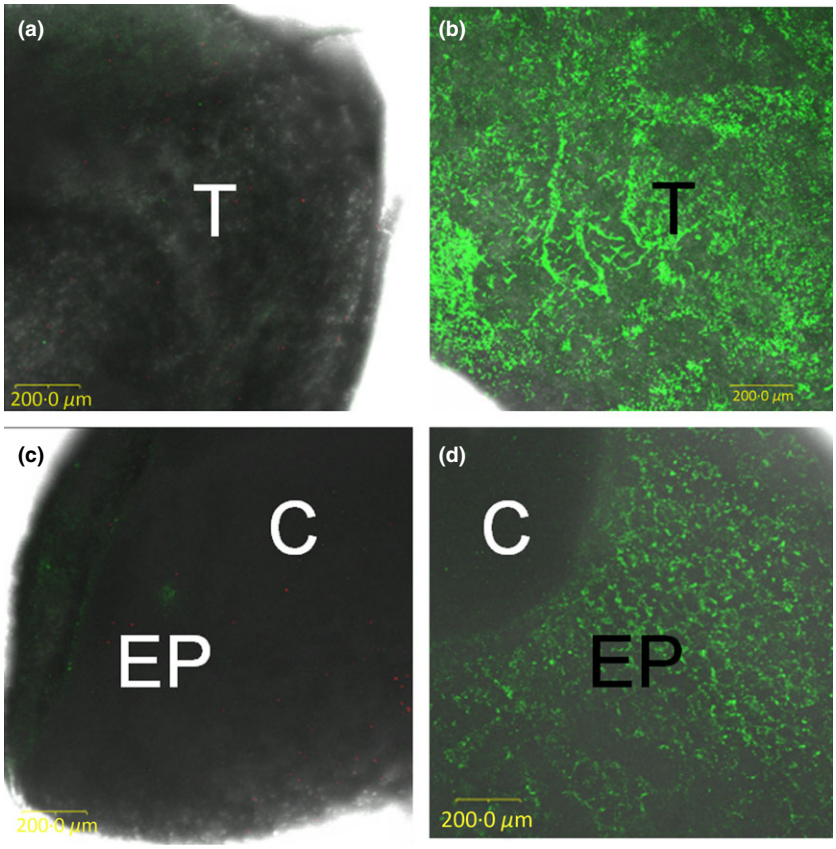


Figure 3 *In situ* immunofluorescence: CGMMV-specific antibodies were used to detect the virus in melon seeds. (a) CGMMV-free seed coat, (b) CGMMV-infected seed coat, (c) inner tissue of a CGMMV-free seed, (d) inner tissue of a CGMMV-infected seed. T, testa; EP, endopleura; C, cotyledon.

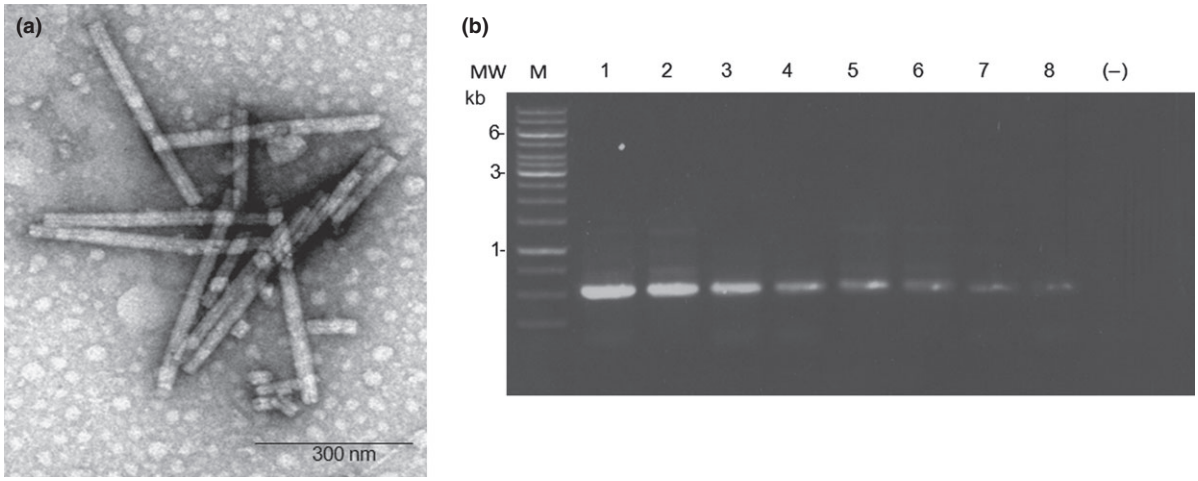


Figure 4 Detection of CGMMV in 40 g batches of infected melon seeds following treatment. (a) Virions isolated from infected seeds subjected to the heat treatment, (b) detection of CGMMV by RT-PCR for the viral CP. M, marker; lanes 1–2, untreated, infected seed (positive control); lanes 3–4, infected seed subjected to the TSP treatment; lanes 5–6, infected seed subjected to the heat treatment; lanes 7–8, infected seed subjected to the combined treatment; (–) negative PCR control.

Table 1 Summary of the effectiveness of seed disinfection treatments in the elimination of *Cucumber green mottle mosaic virus* (CGMMV)

| | | Seed treatment | | | |
|---|---|---|---|---|---|
| Analysis | Amount of seeds | Control (untreated) | Chemical (10% TSP) | Heat (72°C, 72 h) | Combined treatment (10% TSP+heat 72°C, 72 h) |
| ELISA | 40 g | + | + | + | + |
| | 100 seeds | + | + | + | + |
| Fluorescence labelling | 10 seeds | + | + | + | + |
| TEM | 40 g | + | + | + | + |
| RNA | 40 g | + | + | + | – |
| PCR for CP-MP | 40 g | + | + | + | + |
| | 100 seeds | + | + | + | + |
| LD-PCR | 40 g | + | + | + | – |
| | 100 seeds | + | + | – | – |
| Biological test | 40 g | + | + | + | + |
| | 100 seeds | + | + | + | + |

+, presence of CGMMV; –, absence of CGMMV.

infected plants/4 tested plants, 3/4, 3/4 and 4/4); the 10% TSP treatment (2 infected/4 tested plants) and the heat treatment (2 infected/4 tested plants, 2/4, 3/4 and 4/4). A significantly lower level of infectivity (average = 27%) was detected in the replicates of the combined treatment (1 infected plant/4 tested plants, 1/3, 0/3 and 2/4) (Table 1).

The viral RNA extracts from batches of 100 seeds (small-scale) were highly infective in most of the susceptible plants. The extracts from untreated infected seeds (positive control) had a 100% infectivity rate (3 infected plants/3 tested plants, 3/3, 4/4, 3/3). Among the plants inoculated with extract from seeds subjected to the 10% TSP treatment, 83·3% infectivity was observed (3/3, 2/3, 2/3, 3/3). Among plants inoculated with extract from heat-treated seeds, 66·6% infectivity was observed (2/3, 3/3, 1/3, 2/3) and 31·5% infectivity was observed among plants inoculated with extract from seeds subjected to the combined treatment (1/4, 0/3, 2/3, 1/3) (Table 1).

The inability of the three seed treatments to eliminate the infectious capability of CGMMV-contaminated seeds encouraged further investigation of the location of the virus within the seed tissues. To that end, the *in situ* detection of the CGMMV-CP was carried out in treated and untreated contaminated seeds (Fig. 5). Immunofluorescence labelling was visualized by confocal microscopy. This revealed the presence of strong fluorescence signals in the seed coats and inner tissues, possibly the endopleura and the endosperm, following the different treatments, including the combined treatment (Fig. 5c,d).

## Discussion

Since 2007, CGMMV has been identified in different parts of Israel, including remote areas. The virus has been found in trellised cucumber and melon growing in greenhouses and watermelon growing in open fields (Reingold *et al.*, 2013). This widespread CGMMV epidemic encouraged investigation of the role of seeds as a source of primary inoculum. Several disinfection methods based on heat (Kim *et al.*, 2003) or chemical treatment (e.g. 10% TSP; Rast & Stijger, 1987; Herrera-Vásquez *et al.*, 2009) have been reported to provide satisfactory control of tobamoviruses in cucurbit (Kim *et al.*, 2003) and solanaceous seeds such as pepper (Rast & Stijger, 1987) and tomato (Cordoba-Selles *et al.*, 2007). However, the failure of these disinfection treatments, particularly the chemical treatments, to control CGMMV in commercial greenhouses and fields in Israel suggested that, in some cases, the virus may remain infectious even after treatment.

This tolerance could be explained by the preservation of the virus in the inner tissues of the seeds (Rast & Stijger, 1987; Genda *et al.*, 2011). This possibility has also been confirmed indirectly in recent studies that have reported internal changes in seed morphology following infection: development of hair-like tissues on melon seed coats (Lee *et al.*, 2012b) and enlargement of the space between the endopleura and the endosperm (Lee *et al.*, 2012a). These morphological and physiological changes may involve more than the external seed coat absorption of CGMMV and thus support a new supposition that CGMMV is present in the inner tissues of the seed and interacts with the cells of those tissues.

At present, most of the techniques for detecting CGMMV in seeds rely on the serological method ELISA (Clark & Adams, 1977; Koenig, 1981) and amplification-based methods, specifically RT-PCR and quantitative real-time RT-PCR (qRT-PCR) (Henson & French, 1993; Schoen *et al.*, 1996; Fang *et al.*, 2002). ELISA is considered to be a robust technique (Sutula *et al.*, 1986) and it enables the detection of viral CP subunits (Webster *et al.*, 2004), but it suffers from two main limitations. The first is the problem of false negatives; the specificity and avidity of the antibodies used for the analysis can vary and that variation may be particularly important when dealing with small amounts of infected seeds in a large seed lot. According to the International

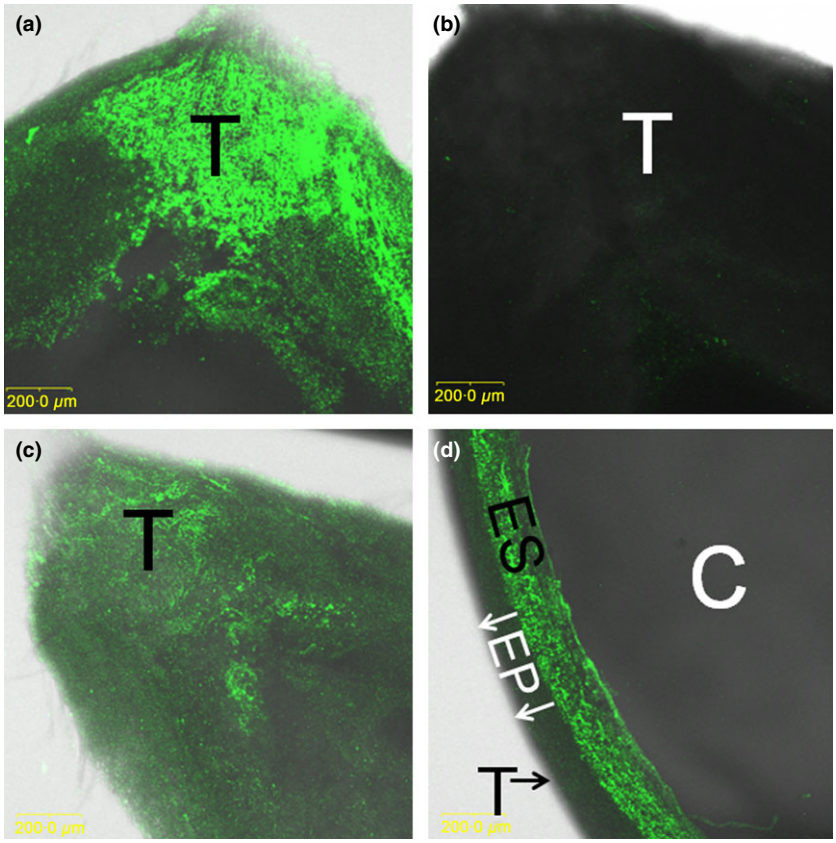


Figure 5 *In situ* immunofluorescence: CGMMV-specific antibodies were used to detect the virus in treated melon seeds. (a) CGMMV-positive seed coat, (b) CGMMV-free seed coat, (c) CGMMV-infected seed coat observed among seed that had received the combined (chemical + heat) treatment, (d) inner tissue from a CGMMV-infected seed that had received the combined treatment. T, testa; EP, endopleura; ES, endosperm; C, cotyledon.

Seed Testing Association (ISTA), proper testing requires 20 batches of 100 seeds each (2000 seeds in total) and such an analysis will have a detection threshold of 1:99 (1 infected seed: 99 healthy seeds). There is often a low virus titre following the conventional chemical treatment and extensive washes performed during the production of commercial seeds, which help to bring the viral concentration below the detection threshold, although viable viral particles still exist (Sutula *et al.*, 1986; Tuitert, 1990). The second limitation of the ELISA method is the problem of false positives and, in some instances, correlation between the ELISA results and biological significance can be problematic (Sutula *et al.*, 1986; Tuitert, 1990). The possibility of obtaining false-positive or false-negative ELISA results may lead to situations in which growers purchase treated seed lots that may be contaminated. Moreover, the inability to draw a direct link between diagnostic results and the degree of virus infectivity is a major obstacle even when more sensitive molecular-based methods such as RT-PCR or qRT-PCR (Henson & French, 1993; Schoen *et al.*, 1996; Fang *et al.*, 2002) are used.

In order to provide a solution for the challenging problem of how to identify CGMMV infectivity potential, a protocol was assembled that included serological, molecular and morphological methods together with a biological assay, and allowed the validation of the efficacy of seed disinfection treatments (Figs 1 & S1). To extend the utility of commonly accepted techniques such as ELISA (Antignus *et al.*, 1990), RT-PCR (Kim *et al.*, 2003) and qRT-PCR (Hongyun *et al.*, 2008), additional parameters related to viral infectivity were measured and calibrated using CGMMV-infected melon and cucumber seeds.

The combined protocol was designed to deal with two different quantities of seed: (i) a smaller amount of 100 seeds, which requires more sensitive methods such as the LD-RT-PCR calibrated in this study, and allows the analysis of the entire viral genome integrity (Figs 1, left path & 2b2); and (ii) a larger amount (40 g) of seeds, which allows the use of more robust methods that rely on highly concentrated samples, obtained by virion purification, that serve as starting material for sequential analyses (Fig. 1, right path). It should be noted that the larger 40 g protocol provides a wider examination of seed samples and is more appropriate for the evaluation of new seed disinfection treatments. The smaller scale protocol may be more appropriate for the examination of seed lots before they are released to the market.

The results here clearly demonstrate that two common seed treatments, 10% TSP and heating to 72°C for 72 h, do not fully inactivate CGMMV in seeds. Each of the detection methods used in this study, ELISA, TEM, RT-PCR and the complementary biological assay, detected the presence of the virus in treated seeds (Table 1). Based on these results, it is concluded that these common seed treatments do not provide effective control of CGMMV in melon and cucumber seeds. According to the results of the biological assay, the combined treatment of 10% TSP for 30 min followed by 72°C for 72 h is more effective than either treatment used alone, but even so, ELISA and RT-PCR both detected the presence of CP subunits in seeds subjected to the combined treatment.

Interestingly, *N. benthamiana* was found to be the preferable test plant, as it develops CGMMV symptoms more rapidly and clearly than other plant species (Fig. 2c). Viral infection could be confirmed in *N. benthamiana* within 5–7 dpi. The high sensitivity of *N. benthamiana* to different plant viruses has been well described previously (Voinnet *et al.*, 1999; Yang *et al.*, 2004; Senthil *et al.*, 2005).

Comparison between the combined protocol and a single analysis (ELISA, RT-PCR or qRT-PCR) commonly used in official diagnostic laboratories, demonstrated that the combined protocol provides more accurate and reliable results regarding the efficacy of the disinfection treatments. For instance, ELISA-negative seed lots may be capable of inducing symptoms on test plants in a biological assay (Haase *et al.*, 1989; Klerks *et al.*, 2001; Menzel *et al.*, 2003). These findings demonstrate the importance of using several techniques to test a single seed lot.

The complexity of the efficacy of seed disinfection treatments is also reflected in the diverse patterns of infectivity observed when treatments are applied on different seed lots; a treatment may be fully effective on one lot and ineffective on a different lot. This inconsistent efficacy can be explained by the presence of the virus in inner tissues of the seed, a situation that occurs irregularly in seed lots (Broadbent, 1965). The presence of the virus in inner tissues could be due to transmission by pollination and subsequent infection of the embryo, but that phenomenon has not been observed for tobamoviruses (Hull, 2002; Genda *et al.*, 2011). On the other hand, in cases of early infection, a high titre of virus can be detected in the fruits, and the formation of seeds in this highly infected environment can lead to the accumulation of virus in the inner parts of the seed (Chitra *et al.*, 1999).

Based on the results presented here, all the tested seed disinfection treatments (heat, TSP and a combination of the two) were found to be insufficient for eliminating the infectivity of CGMMV from contaminated seed lots; the use of the combined protocol prevents misinterpretation regarding the efficacy of the examined treatments (Duhyun & Jungmyung, 2000; Kim *et al.*, 2003) by forcing a dipper examination of the seeds.

The ideal approach for disease control should be based on a sensitive and accurate diagnostic analysis of commercial seed lots to guarantee the use of tobamovirus/CGMMV-free seed. Despite the reliability of the biological assay, this technique, when it stands alone, may suffer from false negative results, especially when dealing with low virus titre. Moreover, the biological assay requires at least 7–9 days post-inoculation, greenhouse space, monitoring environment and pest management control. Hopefully in the near future, application and improvements of LD-RT-PCR for integrity genome detection could join the classical biological assay and advance the detection potential.

Meanwhile, CGMMV-contaminated seed lots should be eliminated until new protocols or methods for efficient disinfection are discovered. Avoiding early infection through the use of CGMMV-free seeds and effective seed treatments will help to decrease the rate of early infection and minimize yield losses.

In conclusion, commercial seed treatments need to be re-examined regularly, in order to verify their efficacy. It is hypothesized that the efficacy of seed treatments may be influenced by the percentage of infected seeds in the treated lot which may be affected by several parameters: (i) CGMMV infection within the parental lines; (ii) the number of CGMMV-infected plants in the seed production area; and (iii) the time and mode of inoculation within the seed production area, i.e. whether the inoculation proceeded by mechanical means (on mature or young plants) or via CGMMV-contaminated pollen. In order to properly evaluate the feasibility and efficacy of cucurbit seed disinfection techniques for the control of CGMMV, further study directed toward the identification of the mode of virus preservation and seed transmission, as well as the location of the virus within the seed tissues, is required.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Figure S1** Protocol for the detection of *Cucumber green mottle mosaic virus* (CGMMV) infectivity in large or small scale of cucurbit seeds.