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Resistance to *Cucumber green mottle mosaic virus* in *Cucumis sativus*

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Abstract *Cucumber green mottle mosaic virus* (CGMMV) is a severe threat for cucumber production worldwide. At present, there are no cultivars available in the market which show an effective resistance or tolerance to CGMMV infection, only wild *Cucumis* species were reported as resistant. Germplasm accessions of *Cucumis sativus*, as well as *C. anguria* and *C. metuliferus*, were mechanically infected with the European and Asian strains of CGMMV and screened for resistance, by scoring symptom severity, and conventional RT-PCR. The viral loads of both CGMMV strains were determined in a selected number of genotypes using quantitative RT-PCR. Severe symptoms were found following inoculation in *C. metuliferus* and in 44 *C. sativus* accessions, including *C. sativus* var. *hardwickii*. Ten *C. sativus* accessions, including *C. sativus* var. *sikkimensis*, showed intermediate symptoms and only 2 *C. sativus* accessions showed mild symptoms. *C. anguria* was resistant to both strains of CGMMV because no symptoms were expressed and the virus was not detected in systemic leaves. High amounts of virus were found in plants showing severe symptoms, whereas low viral amounts found in those with mild symptoms. In addition, the viral amounts detected in plants which showed intermediate symptoms at 23 and 33 dpi, were significantly higher in plants inoculated with the Asian CGMMV strain than those with the European strain. This difference was statistically significant. Also, the amounts of virus detected over time in plants did not change significantly. Finally, the two newly identified partially resistant *C. sativus* accessions may well be candidates for breeding programs and reduce the losses produced by CGMMV with resistant commercial cultivars.

Keywords Real-time RT-PCR · Viral load · CGMMV · Tobamovirus

Introduction

Diseases produced by viruses cause economic losses in commercial cucurbit production around the world (Lovisolo 1980). Among these viruses, *Cucumber green mottle mosaic virus* (CGMMV) represents a major risk in the production of melon, watermelon and cucumber. CGMMV belongs to the genus *Tobamovirus*, family *Virgaviridae* (Adams et al. 2009) and causes systemic mottle and mosaic symptoms on

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cucurbitaceous plant leaves (Kim et al. 2003). In watermelon, the virus causes a pulp deterioration called blood flesh disease (Mandal et al. 2008) and the fruit loses its marketable value. In cucumber, the virus causes deformation and mosaic symptoms (van Koot and van Dorst 1959). It is mechanically, pollen- and seed-transmitted (Liu et al. 2014).

CGMMV was first described infecting cucumber in England by Ainsworth (1935). Its incidence in other countries of the world has increased rapidly during the last decade, possibly through the international seed trade following cucurbit seed crop production in tropical or subtropical countries (Dombrovsky et al. 2017). Also, seed-testing routines for CGMMV may be inadequate, which allows for the rapid and world-wide spread of the virus. Contaminated seeds provide a route for the movement of the virus between countries and its introduction into new areas, and several seed treatments currently used, were found insufficient to eliminate the virus from contaminated seed lots. In addition, like other tobamoviruses, CGMMV can survive for a long time on plant debris from infected crops (Reingold et al. 2015). Therefore, control depends on early monitoring, awareness of the farmers, and an appropriate crop management, but even implementing these measures, success is not guaranteed. (Reingold et al. 2016).

Worldwide CGMMV isolates are grouped in two major clusters based on biological differences and genome sequences: A first cluster (I) constitutes the European strain and includes most isolates from France, the Netherlands, and Uzbekistan. A second cluster (II) is formed by isolates from Asian countries such as Japan and South Korea (Crespo et al. 2017). Spain is currently the first country where both these strains have been described co-infecting the same crops and in the same region (Crespo et al. 2017).

Commercial tomato and pepper hybrids that carry virus-resistance genes successfully control tobamoviruses in solanaceous crops. But in the case of CGMMV, there is an urgent need for resistant cultivars in cucumber, with restricted virus movement, replication and symptom development. However, currently cucumber varieties resistant to CGMMV are hardly available. Efforts have been made to produce transgenic resistance in the experimental plant *Nicotiana benthamiana* (Kamachi et al. 2007), in watermelon (Park et al. 2005; Lin et al. 2012) and in melon (Ali et al. 2012). Alternatively, recent reports showed that *Stenotrophomonas maltophilia* HWS exhibits a good biocontrol against the virus by increasing the expression of defense response genes (Li et al. 2016) and an attenuated strain used as a biocontrol agent reduced yield losses in cucumber (Slavokhotova, et al. 2016).

The development of resistant varieties through conventional breeding could offer a good solution to this disease which continues to escalate. Resistance to CGMMV has been reported only in melon and in wild *Cucumis* species (Rajamony et al. 1987, 1990a, b). In *Cucumis*, several wild species of African origin are classified as resistant. One of them is *C. anguria* L., which has one dominant gene that confers resistance to CGMMV (den Nijs 1982). *C. figarei* has been described as immune, while other resistant types like snapmelon "Kachri" and other *Cucumis* sp. are symptomless carriers (Pan and More 1996). In melon, a new source of resistance to CGMMV-SH has been identified in accession "Chang bougi" (Sugiyama et al. 2006). In cucumber, *C. sativus* var. hardwickii (R.) Alef. has been used to introduce resistance to CGMMV (Carnide and Barroso 2006).

In the present paper we used a real-time RT-PCR test to determine the viral loads of the European and the Asian strain of CGMMV. An isolate of each strain was mechanically inoculated in a collection of 56 *C. sativus* accessions. We report on the evolution of symptom expression for both virus strains in all accessions, and that of the viral loads on a representative number of plants.

Materials and methods

Plant material

Accessions from a *C. sativus* collection were supplied by the Institute for Conservation and Improvement of Valencian Agrodiversity (COMAV-UPV) representing three variants of the species: 50 accessions of *C. sativus* L., 5 of *C. sativus var. hardwickii* (Royle) Gabaev and 1 of *C. sativus var. sikkimensis* (Hook.f.). Some of the accessions were collected by COMAV and others were obtained from exchanges with other germplasm banks (USDA-NPGS and CATIE). The selected accessions were meant to represent the

variability of the full collection. In addition, we tested *C. anguria* L. and *C. metuliferus* E. Mey as African wild species and which were supplied by Seednative (La Iruela, Spain).

Virus sources for mechanical inoculation

In order to investigate differences in resistance, we mechanically inoculated plants with one of two isolates that represent the two different strains of CGMMV: the strain of European origin (CGMMV-SP) (GeneBank GQ411361) and that of Asian origin (CG-SPCu16), the latter as determined by Crespo et al. (2017). Although both strains differ in their genome sequences, they do not show any biological difference in terms of systemic symptoms expressed on leaves of infected cucumber. Both strains showed severe mosaic symptoms and blisters. However, they behaved differently in *Chenopodium amaranticolor* in which local lesions appeared when Asian-like strains were inoculated (Crespo et al. 2017). Both strains were isolated at IFAPA from cucumber crops in Almeria (Spain) in 2009 and 2015, respectively. They had been isolated after three passages into *Gomphrena globosa* L., then propagated in cucumber (cv. Cumlaude), and stored at − 80 °C until used for the mechanical inoculation. Before use in screening the cucumber accessions, the isolates were propagated after mechanical inoculation in cucumber (cv. Cumlaude). Infected plants were grown in an insect-proof greenhouse where temperature was partially controlled (25–30 °C). Approximately 3 weeks after inoculation, plants that showed typical symptoms of virus infection were used as the virus source.

Mechanical inoculation

For each virus isolate, 0.5 g of tissue was taken, 5 weeks after sowing and from the second leaf down from the plant apex which displayed CGMMV symptoms. The tissue was homogenized in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.0) and inoculated mechanically by rubbing 150 µl of the extract onto leaves dusted with carborundum powder. At least ten plants of each species were inoculated with each virus isolate and the experiment was repeated twice, each time with two repetitions, during the winter (January–March) and in spring–summer period (May–July) of 2017. All inoculated plants were maintained in an insect-proof greenhouse under controlled conditions.

Evaluation of symptoms and detection of the virus

Inoculated plants were evaluated for the expression of CGMMV symptoms at 23, 33 and 47 days post inoculation (dpi), using the following scale: 0 (symptomless), 1 (mild symptoms, as initial mottle mosaic on leaves), 2 (intermediate symptoms, as evident leaf mottle mosaic on leaves) and 3 (severe symptoms as mottle mosaic, interveinal chlorosis, and blistering in leaves, and distortion and mosaic in fruits). The presence of the virus was analyzed at 15 dpi using a conventional RT-PCR reaction using the primers that amplify part of the coat protein-coding region as described in Crespo et al. (2017).

From plants of selected accessions, 0.2 g tissue was removed from the second leaf (not inoculated, and possibly representing systemic infection) from the apex at 23 (plants with 3 new leaves formed), 33 (plants with 5 new leaves) and 47 (plants with 8 new leaves) dpi for analysis by real-time RT-PCR which was developed for the detection and quantification of CGMMV. The infected leaf tissue of each plant was ground to a fine powder in liquid nitrogen in a pestle and mortar and placed in a sterile microcentrifugue tube. Total RNA was extracted with Trizol reagent (Invitrogen). The resulting pellet was resuspended in 50 µl DEPC-treated water and stored at − 80 °C. RNA was quantified with a ND-2000c Spectrophotometer (NanoDrop Technologies) and diluted to a final concentration of 50 ng/µl. Real time RT-PCR reactions were set up in 96-well reaction plates using TaqMan One Step RT-PCR Master Mix (Applied Biosystems). One microlitre aliquots were used as templates in the RT-PCR reactions of 20 µl, containing 10 µl Master Mix, 1 µl forward primer, 1 µl reverse primer, 0.5 µl probe, 1 µl total RNA and 6.5 µl DEPC-water. Following Chen et al. (2008), the primers and probe for the TaqMan assay were 5′-GCATAGTGCTTTCCCGTTCAC-3′ (sense) at positions 6285–6305nt and TGCAGAATTACTGCCCA TAGAAAC-3′ (antisense) at positions 6362–6385nt. The probe was 5′-CGGTTTGCTCATTGGTTT GCGGA-3′ at positions 6316–6338nt, labeled with 6-carboxyfluorescein (FAM) and the 3′end was labeled with N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA). The primers and probe for

amplification of the internal controls were: primers CUC18S-For (5′-GGCGGATGTTGCTTTAAGGA-3′) and CUC18S-Rev (5′-GTGGTGCCCTTCC GTCAAT-3′; probe CUC18S-Ana (5′-TCCGCCAG CACCTTATGAGAAATCAAAGTC-3′) labeled with JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein) as the 5′terminal reporter dye and with BHQ1 (Black Hole Quencher One) as the 3′ quencher dye (Gil-Salas et al. 2009). Three technical replications were performed per sample and the tests were run on ABI Prism 7000 DNA sequence detection system (Applied Biosystems) as follow: 10 min at 50 °C, 1 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Relative accumulation of CGMMV in the plants was calculated by the comparative Ct (cycle threshold) method, using as an endogenous reference gene a fragment within the 18S ribosomal region of the cucurbit mitochondrial genome (Gil-Salas et al. 2009), and as a calibrator, untreated control (uninfected cucumber). In this way, with RT-PCR we obtained amplified fragments of the expected size using real-time PCR primers (virus and internal control). The expected PCR products were cloned and sequenced. The sequence identity of the internal control and the virus was confirmed after comparison with GQ411361 and AF206894 (*C. sativus* 18S ribosomal RNA gene) GenBank sequences. The relative accumulation of the virus from collected samples was calculated using the $2^{-\Delta\Delta Ct}$ formula. Here, ΔCt is the difference between the Ct of each sample (mean of the three technical repetitions) and the Ct of the internal control (mean of the three technical repetitions) (ΔCt = Ct sample − Ct internal control), and ΔΔCt is the difference between ΔCt and ΔCt calibrator (ΔΔCt = ΔCt − ΔCt calibrator). Finally, $2^{-\Delta\Delta Ct}$ represents the amount of target, normalized to the endogenous reference and relative to the calibrator (Livak and Schmittgen 2001; Sáez et al. 2016).

Data analysis

Resistance was evaluated as the response of the host plant to virus infection estimated from symptom severity in all inoculated plants, and from the viral titer in a selected number of genotypes (12 accessions representing *C. sativus*; *C. anguria* and *C. metuliferus*). The interaction effects from inoculated strain of virus, dpi and symptoms were investigated using general linear model statistics. All effects were tested at the 5% significance level. Analyses were performed using Statistics 9.1 statistical software.

Results

Symptom expression

Single inoculations in cucumber with CGMMV isolates CGMMV-SP and CG-SPCu16 that represent the European and the Asian strain, respectively, produced three clearly differentiated levels of symptoms: mild symptoms (soft mottling), intermediate symptoms (limited to evident mottle-mosaic on leaves), or severe symptoms, that showed as leaf mottle mosaic, interveinal chlorosis, and blistering in leaves, with distortion and mosaic in fruits (Fig. 1). The time of appearance of these symptoms in individual plants was variable, but all of the replicate plants of each accesion showed similar symptoms at 23 dpi. We observed no change in the symptoms at 33 and 47 dpi. Most of the 56 tested *C. sativus* accessions were highly sensitive to the mechanical transmission of CGMMV, 44 of which developing severe symptoms including *C. sativus* var. *hardwickii*. Only 2 accessions showed mild symptoms (BGV001358, from Calcuta, and CGN19818 from Dzampur, India) and 10 accessions from India and Spain showed intermediate symptoms up to the end of the test period, including *C. sativus* var. *sikkimensis* (Table 1). Finally, regarding to the African wild species, *C. anguria* was symptomless and tested negative in conventional RT-PCR, whereas *C. metuliferus* showed severe symptoms and tested positive (Fig. 2).

Identical results were obtained in two repeat experiments under the same conditions and following the methodology described above, one during the winter period (January–March 2016–2017) and the second one in the spring–summer period (May–July 2017).

CGMMV viral loads

All inoculated plants, except for *C. anguria*, tested positive by the conventional RT-PCR test. Plants from a selection of accessions (marked in Table 1) that covered the three types of symptoms when inoculated with the CGMMV strains, were analyzed with real-time RT-PCR in samples collected at 23, 33 and 47 dpi

Fig. 1 Leaf symptoms on cucumber at 23 days post-inoculation with CGMMV-SP: (**a**) mild symptoms, (**b**) intermediate symptoms, (**c**) severe symptoms

(see Table as Supplementary Material). The accessions selected to represent the range of symptoms, consisted of 5 genotypes that showed severe, 5 that showed intermediate, and 2 that showed mild symptoms. The results from the accumulated analysis of variance on the real-time RT-PCR data showed a significant effect with respect to the level of expressed symptoms ($P < 0.001$). This suggested that the viral titer was positively correlated with the degree of symptoms expressed in the inoculated plants. Moreover, the difference in the normalized amount of CGMMV ($\log 2^{-\Delta\Delta Ct}$) when the cucumbers had been inoculated with European or Asian genotypes was also statistically very significant ($P < 0.001$; Table 2). In addition, the amounts of virus of CG-SPCu16 (Asian strain) were higher in accessions with intermediate symptoms at 23 and at 33 dpi ($P < 0.001$ and $P < 0.05$, respectively), when compared with those infected with CGMMV-SP (Fig. 3). On the other hand, the amounts of virus detected over time (dpi) regarding to the symptom observed, did not change significantly (Table 2).

Discussion

We tested a collection of cucumber germplasm accessions against CGGMV, and most genotypes were very sensitive to the virus after mechanically inoculation. Moreover, the high susceptibility observed along the whole range of diversity of this species confirmed that this virus represents a major threat to cucumber cultivation. Out of 58 accessions evaluated, 10 produced only intermediate level symptoms, suggesting a certain level of tolerance. Interestingly, we also have identified 2 accessions (BGV001358 and CGN19818) that showed only mild symptoms upon infection with CGMMV, and that accumulated a low viral titer. Resistance is an important factor that determines concentrations of virus in several virus-cucurbita host plant pathosystems. This happens in the case of *Cucurbit yellow stunting disorder virus* and *Watermelon mosaic virus* in melon (Marco et al. 2003; Díaz-Pendón et al. 2005), and *Papaya ringspot virus* in squash and watermelon (Pacheco et al. 2003). The plants from the two accessions, both originated from India, produced mild symptoms after inoculation with CGMMV, and may have a higher level of resistance. This resistance could be related with the co-evolution of host and pathogen in this part of the world.

Different cultivars may accumulate different amounts of virus when infected with CGMMV, and those plants that have restricted viral load could be regarded as potential sources of resistance (Cech and Branisovâ 1976). Other reports of CGMMV resistance tests in vegetable accessions have enabled to select only a limited number of interesting cultivars. Of 345 cultivars tested against CGMMV, all produced mosaic symptoms in leaves, except for only one, named "Hanboksamcheok", which showed mild mosaic symptom in a field test (Ko et al. 2004).

C. metuliferus was found very susceptible to CGMMV (Table 1, Fig. 2). In contrast, the wild species *C. anguria* could be a potential source of resistance to CGMMV due to the fact that it did not develop any symptoms following inoculation and that it remained negative for the virus when tested with

Table 1 Response of *Cucumis sativus* accessions, *C. anguria* and *C. metuliferus*, to mechanical inoculation with CGMMV

Accession[a]	Origin	Species; cultivar	CGMMV-SP			CG-SPCu16		
			23 dpi	33 dpi	47 dpi	23 dpi	33 dpi	47 dpi
BGV000040	Gea de Albarracín, Teruel, Spain	*Cucumis sativus*	3[b]	3	3	3	3	3
BGV000377	Jimena de Líbar, Málaga, spain	*Cucumis sativus*	3	3	3	3	3	3
BGV000408	Los Barrios, Cádiz, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV000460*	Alcaudete, Jaén, Spain	*Cucumis sativus*	2	2	2	2	2	2
BGV000467	Martos, Jaén, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV000469	Guéjar de la Sierra, Granada, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV000503	Aracena, Huelva, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV000518*	Laujar de Andarax, Almería, spain	*Cucumis sativus*	2	2	2	2	2	2
BGV000524*	Olivar, Granada, Spain	*Cucumis sativus*	2	2	2	2	2	2
BGV001310	Pola de Siero, Asturias, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV001358*	Calcuta, India	*Cucumis sativus*	1	1	1	1	1	1
BGV001774*	Torelló, Barcelona, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV002473	San Pedro, Gomera, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV002505	El Tablado, La Palma, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV003267	Canadá	*Cucumis sativus*	3	3	3	3	3	3
BGV003366	Hontalbilla, Segovia, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV003371	Venta de Baños, Palencia, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV003372	Simancas, Valladolid, Spain	*Cucumis sativus*	2	2	2	2	2	2
BGV003688	Alcalá de Júcar, Albacete, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV003713	Molinos de Papel, Cuenca, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV004001	Hoyos, Cáceres, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV004308	Churra, Murcia, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV004532*	La Habana, Cuba	*Cucumis sativus*	2	2	2	2	2	2
BGV004893	Ademuz, Valencia, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV004925	Castellón, Spain	*Cucumis sativus*	2	2	2	2	2	2
BGV004981	Beneixama, Alicante, Spain	*Cucumis sativus*	3	3	3	3	3	3
BGV010299	Guadalajara, Spain	*Cucumis sativus*	2	2	2	2	2	2
BGV010350	Calzada de Calatrava, Ciudad Real, Spain	*Cucumis sativus*	3	3	3	3	3	3
CGN23417	India	*Cucumis sativus*; Kheera	3	3	3	3	3	3
CGN23632	India	*Cucumis sativus*; Allahabad Rainy Season	3	3	3	3	3	3
CGN22295	India	*Cucumis sativus*; ARC1	3	3	3	3	3	3
CGN21675	India	*Cucumis sativus*; Poona Kheera	3	3	3	3	3	3
CGN20909	India	*Cucumis sativus*; Hanzil	3	3	3	3	3	3
CGN21584	India	*Cucumis sativus*; Poona Kheera	3	3	3	3	3	3
CGN19819	India	*Cucumis sativus*; Puneri Klura VIR 2803	3	3	3	3	3	3

Table 1 continued

Accession[a]	Origin	Species; cultivar	CGMMV-SP			CG-SPCu16		
			23 dpi	33 dpi	47 dpi	23 dpi	33 dpi	47 dpi
CGN23089	India	*Cucumis sativus*; 1*1751 P1197087*	2	2	2	2	2	2
CGN22280	India	*Cucumis sativus*; Shuei Huang Kua VIR 1311	3	3	3	3	3	3
CGN19817*	India	*Cucumis sativus*; Cucumber Medium VIR 3136	3	3	3	3	3	3
CGN24668*	India	*Cucumis sativus var. sikkimensis*; *IC 4230 sikkim cucumber*	2	2	2	2	2	2
CGN22281	India	*Cucumis sativus*; *Long Green WIR2923*	2	2	2	2	2	2
CGN23411	India	*Cucumis sativus*; Khira Cheshuichatyi Indiiskii oguretc khira sosa	3	3	3	3	3	3
CGN23423*	India	*Cucumis sativus*; *JL-2 Dhillon*	3	3	3	3	3	3
CGN22986*	India	*Cucumis sativus*; *Smallgreen Bogarnyi*, *VIR 1423*	3	3	3	3	3	3
CGN19748	India	*Cucumis sativus*; *Khira*; *PI271328*: *No. 69*	3	3	3	3	3	3
CGN21585	India	*Cucumis sativus*; *Saharanpur*	3	3	3	3	3	3
CGN22297	India	*Cucumis sativus*; *JL-8 Dhillon*	3	3	3	3	3	3
CGN19818*	India	*Cucumis sativus*; *Dzampur*; *VIR 3066*	1	1	1	1	1	1
CGN23002*	India	*Cucumis sativus var. hardwickii*; *JL-14 Dhillon*	2	3	3	2	3	3
CGN24497	India	*Cucumis sativus* var. *hardwickii*	3	3	3	3	3	3
CGN24666	India	*Cucumis sativus* var. *hardwickii*	3	3	3	3	3	3
CGN24495	India	*Cucumis sativus* var. *hardwickii*	3	3	3	3	3	3
CGN24667	India	*Cucumis sativus* var. *hardwickii*	3	3	3	3	3	3
CUS 124	China	*Cucumis sativus*	3	3	3	3	3	3
CUS 696	Japan	*Cucumis sativus*	3	3	3	3	3	3
CUS 260	Korea	*Cucumis sativus*	3	3	3	3	3	3
CUS 482	Mongolia	*Cucumis sativus*	3	3	3	3	3	3
		Cucumis anguria	0	0	0	0	0	0
		Cucumis metuliferus	3	3	3	3	3	3

[a]All the assayed accessions were obtained from the germplasm collections of the Institute for Conservation and Improvement of Valencian Agrodiversity, Spain (COMAV-UPV)

[b]Range of symptoms scored in 10 plants per accession at three time points following mechanical inoculation with CGMMV-SP and CG-SPCu16 isolates according to the following scale: 0, symptomless; 1, mild symptoms; 2, intermediate symptoms and 3, severe symptoms or plant death. In all cases the percentage of plants showing symptoms of CGMMV after the mechanical inoculation was 100%

*Accessions included in real-time RT-PCR analysis

real-time RT-PCR, suggesting that this species is not susceptible to CGMMV. Although attempts to cross *C. anguria* with either cucumber or melon have failed so far (Deakin et al. 1971; Kho et al. 1980; Kroon et al. 1979), there appears to be a possibility that some kind of hybridization can be achieved between *C. sativus* and *C. anguria* (den Nijs 1982).

In many virus-host plant pathosystems, concentrations of virus in infected leaves have been found to rise rapidly and subsequently decline (Matthews 1991).

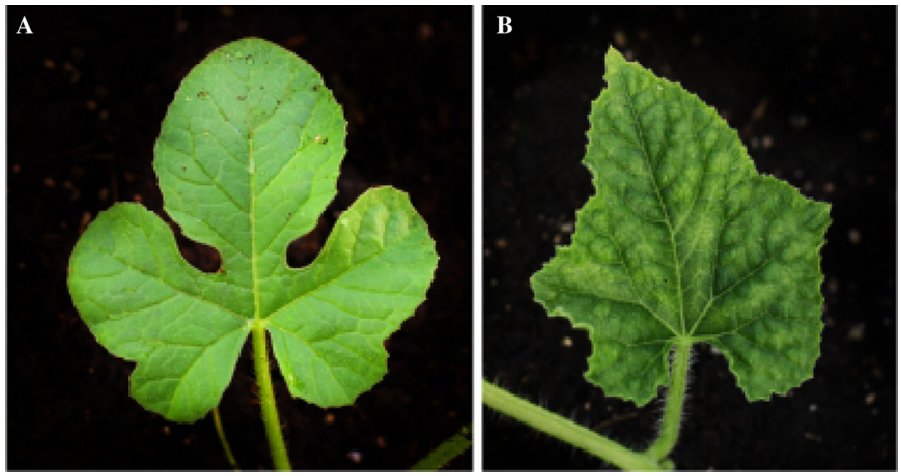

Fig. 2 *C. anguria* (**a**), without visible symptoms, and *C. metuliferus* (**b**), showing severe symptoms at 23 days post-inoculation with CGMMV-SP. Identical results were obtained following inoculation with CG-SpCu16

Table 2 Accumulated analysis of variance results of the normalised amount (log $2^{-\Delta\Delta CT}$) of CGMMV

Change	Degrees of freedom	Sum of squares	Mean square	Variance ratio	*F* probability
Accession[a]	11	100	9	7.95	0.0000
Symptom[b]	2	66	33	23.50	0.0000
Dpi[c]	2	3	1	1.21	0.3007
Virus isolate[d]	1	8	8	7.14	0.0009
Symptom*dpi	8	77.1	10	6.95	0.0000
Symptom*virus isolate	5	95.1	19	15.72	0.0000
Dpi*virus isolate	5	18.3	4	2.01	0.0817
Residues	117	134	1		
Total	131				

[a]Samples were from 12 cucumber accessions plants (marked in Table 2)

[b]Symptom levels were: mild, intermediate and severe

[c]Samples collected at 23, 33 and 47 days post-inoculation (dpi)

[d]Virus isolates were CGMMV-SP or CG-SPCu-16

Cucumber plants inoculated with *Cucumber vein yellowing virus* accumulated concentrations of the virus during 2 months, but some cultivars showed reductions in concentrations sampled at 45 dpi coinciding with the time of fructification, during which fruits exerts a significant sink demand and become highly competitive with vegetative growth (Pharr et al. 1985; Gil-Salas, et al. 2009). As already suggested over 40 years ago, tolerance to CGMMV may be associated with differences in concentration of virus, although there need not be a direct relationship between virus symptoms and the virus concentration. Therefore, the evaluation of resistance against tobamoviruses should include symptomatic and quantitative virus assays (Cech and Branisovâ 1976). In the present study we found a significant relationship between the expressed symptoms and the concentrations of CGMMV-SP or CG-SpCu16. Furthermore, some plants with very mild symptoms contained significant amounts of the virus, which could enhance the possibilities of further spread in crops. In contrast to what is observed with other cucurbit viruses (Gil-Salas et al. 2009), CGMMV virus load did not change significantly over time (Table 2). In this case, we have

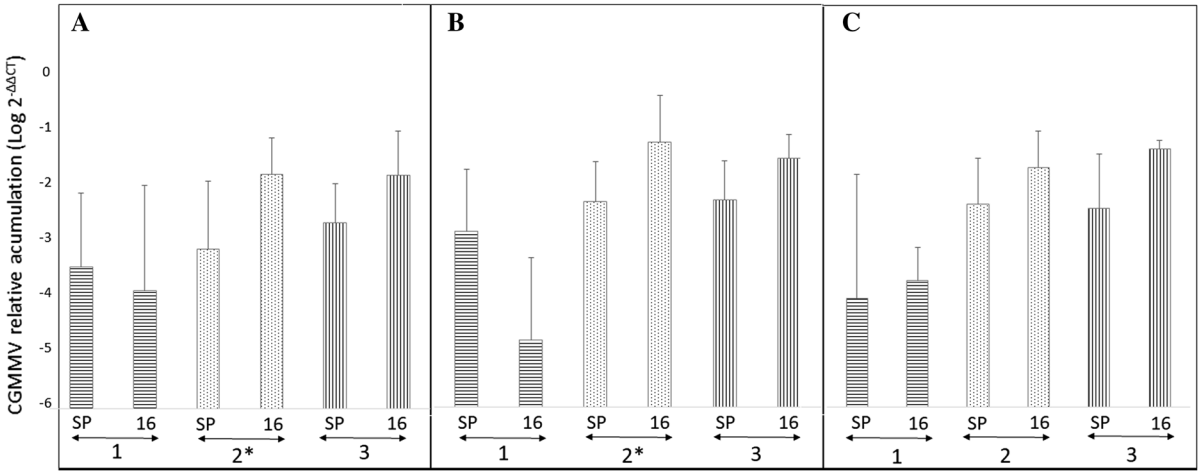

Fig. 3 Error-Bar plots of normalized amounts of CGMMV (log $2 - \Delta\Delta CT$) for the real-time RT-PCR analysis in selected cucumber accessions (marked in Table 2) for days post inoculation (dpi) (**a**: at 23 dpi, **b**: at 33 dpi or **c**: at 47 dpi), symptom (1: mild, 2: intermediate or 3: severe), and virus isolate (SP: from European origin, CGMMV-SP, or 16: from Asian origin, CG-SPCu16); $^*P < 0.01$

not found a relationship between fructification or flowering and a reduction in virus titer.

Taqman real-time RT-PCR has been developed for routine detection of CGMMV and is recommended for use in the field of plant quarantine; this technique, as described by Chen et al. (2008), detects low quantities of virus which would be assist in the prevention and control of the disease caused by this virus. In the present paper we supplied the qRT-PCR test with an internal control based on the cucumber 18S housekeeping gene to normalize the quantification of the viral sequence (Gil-Salas et al. 2009), so that this technique can be used in cucumber breeding programs to estimate the amounts of CGMMV in inoculated plants. We found that the CGMMV-specific primers and probe were able to detect isolates from the European as well as the Asian strain. These strains are genetically and biologically different, yet symptomatology in the field can be similar, and both can be present in the same fields, especially after the recent introduction of the Asian-type CGMMV in many countries of the world (Crespo et al. 2017; Dombrovsky et al. 2017). We found that the overall concentrations of both virus strains in cucumber were different ($P < 0.001$; Table 2). They were similar in plants that were most sensitive and those that showed mild symptoms ($P > 0.05$ in all cases). However, in plants that showed intermediate symptoms, the viral titers of CG-SpCu16 were significantly higher rather than those of CGMMV-SP at 23 and 33 dpi (Fig. 3), which may be explained by the differences between the genome sequences as well as the different reactions in determined host plants following inoculation from both strains as has been previously described (Crespo et al. 2017). This should be taken into account during plant breeding programs, and the susceptibility and sensitivity of candidate accessions should be checked with both strains which have different geographic origins. High seed transmission rates (over 75%) from CGMMV-infected cucumber plants have been reported, and the seed-borne nature of this virus is considered the main cause of spread to new countries and areas (Liu et al. 2014). However, CGMMV is found both on and within the seed coat, so detection in seed batches does not differentiate CGMMV that is contaminating seeds externally from that which will infect the seedling (Reingold et al. 2015). The differential viral accumulation in sensitive and resistant cucumber accessions and differential accumulation of European and Asian strains could also now be investigated with respect to the presence of CGMMV in seeds and its relevance to the risk of transmission in the field.

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