Mechanical transmission of *Tomato leaf curl New Delhi virus* to cucurbit germplasm: selection of resistant sources in *Cucumis melo*

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Abstract

Cucurbits are major crop species, including fruits and vegetables cultivated worldwide that supply essential vitamins and minerals to current diets in developed and developing countries. Viral diseases are main factors affecting cucurbits cultivation. The most widespread and damaging have been aphid-borne viruses belonging to the Potyviridae family. Whitefly-transmitted begomoviruses (Geminiviridae) have been identified more recently in different cucurbit species. A severe outbreak of *Tomato leaf curl New Delhi virus* (ToLCNDV) occurred in pumpkins and melons in the main production area of Southern Spain in 2012-2013. We developed a mechanical inoculation method to facilitate the screening of germplasm against this virus. Mechanical transmission with this method was confirmed in 4 genera and 13 species of the family, including the main crops, cucumber, melon, watermelon and pumpkins, and also crop-related exotic germplasm (landraces and wild species) used for cucurbits breeding. Diversity in the response was observed within and among species. Resistance was identified in melon, within *C. melo* subsp. *agrestis* var. *momordica* and in wild *agrestis* accessions. All the resistant accessions came from India, the country in which this virus was firstly reported. Some of these accessions had been previously reported to be resistant to other viruses and as they are fully crossable to commercial melons are good sources of resistance to develop new melon varieties with resistance to ToLCNDV.

Keywords: *Cucurbitaceae*, *Begomovirus*, ToLCNDV, Melon, Resistance, Breeding

Introduction

Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus (family *Geminividae*) with two circular single-stranded DNA genomes of approximately 2.7 kb each (designated as DNA-A and DNA-B), which are encapsidated in geminate particles (Papidam et al. 1995; Fauquet et al. 2008; Ito et al. 2008). ToLCNDV was first reported on tomato (*Solanum lycopersicum* L.) in north India in 1995 (Srivastava et al. 1995). Subsequently, it has been reported across the country and in other neighboring countries on several plant species, particularly vegetables of the Cucurbitaceae and Solanaceae families, such as cucumber and melon (*Cucumis sativus* L. and *Cucumis melo* L.), watermelon (*Citrullus lannatus* [Thunb.] Matsum. & Nakai), bottle gourd (*Lagenaria* spp), sponge and ridge gourds (*Luffa* spp), bitter gourd (*Momordica charantia* L.), wax gourd (*Benincasa hispida* [Thunb.] Cogn.), pumpkin (*Cucurbita maxima* Duchesne), chilli pepper (*Capsicum* spp) and potato (*Solanum tuberosum* L.) (Chang et al. 2010; Khan et al. 2012; Jyothsna et al. 2013; Bandaranayake et al. 2014).

The first reference of ToLCNDV in Europe comes from Spain. In autumn and spring of 2012-2013, a novel disease was observed in zucchini crops (*Cucurbita pepo* L.) in Murcia and Almeria provinces (Southern Spain). Symptoms included curling and severe mosaic of young leaves, short internodes and fruit skin roughness (Juárez et al. 2014). ToLCNDV was isolated from these symptomatic plants and in fall 2013 it was also found infecting melon and cucumber crops. This virus caused a great impact with catastrophic losses in this horticultural region, and is considered a serious threat to greenhouse and open-field cucurbit crops.

Since ToLCNDV primarily infects tomato and other solanaceous crops, the search for resistance in cucurbits has not been a primary goal in the affected countries. Even though monogenic resistance has been reported in a breeding line of sponge gourd (*Luffa cylindrica* M. Roem.) (Islam et al. 2011), to our knowledge no resistance has been described in the *Cucurbita*, *Cucumis* or *Citrullus* genera.

The availability of an efficient and practical inoculation method is necessary for a successful screening for resistances. Although most ToLCNDV isolates are only naturally transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a persistent manner (Chang et al. 2010), some of them have been shown to be mechanically sap transmitted to their original hosts. A strain of ToLCNDV causing yellow mosaic and leaf distortion

symptoms in potato in India was sap transmitted to *Nicotiana benthamiana* Domin and potato plants (Usharani et al. 2004). A ToLCNDV isolate causing mosaic, leaf curl and puckering in oriental melon (*Cucumis melo* subsp. *agrestis* (Naudin) Greb. var. *makuwa*) of Taiwan, was also reported to be mechanically inoculated to *N. benthamiana* and some cucurbit species, such as oriental melons, *Cucumis melo* subsp. *agrestis* var. *conomon* and *makuwa*, cucumber, bottle gourd, zucchini and sponge gourd. However, no infection was detected after mechanical sap transmission in other cucurbit species, including *Citrullus lanatus*, *Cucumis melo* subsp. *melo* var. *reticulatus* (muskmelon), *Cucumis metuliferus* E. Mey. ex Schrad. and *Cucurbita moschata* Duchesne (Chang et al. 2010). In contrast, another isolate from the same area could not be mechanically transmitted to cucumber (Samretwanich et al. 2000). In addition, a ToLCNDV isolate causing yellow mosaic in sponge gourd from India was sap transmitted to ridge gourd (*Luffa acutangula* Roxb.), sponge gourd and *N. benthamiana* (Sohrab et al. 2013).

In the present work, we report a protocol that ensures efficient and reliable mechanical transmission of a devastating ToLCNDV Spanish isolate. Using this protocol, we demonstrate the wide host range for this isolate including several economically important cucurbit crops. Successful inoculation of ToLCNDV to individual plants is necessary to evaluate the level of resistance of genotypes that may be used in breeding programs. The availability of this highly efficient method for mechanical transmission has facilitated the identification of sources of resistance to ToLCNDV in melon.

Materials and methods

Virus source

Naturally infected zucchini plants collected from Almeria (Southern Spain) showing curling, vein swelling and severe mosaic in young leaves, short internodes, and fruit skin roughness, were used as the original source of inoculum. ToLCNDV infection was confirmed in these plants by PCR using the primers and procedure reported below and the virus was transmitted to zucchini seedlings by virus-free whiteflies, to eliminate any possible contamination with other viruses. Subsequently, ToLCNDV infected zucchini plants were tested, by PCR using specific primers (Picó et al. 2005), for the presence of

Cucumber vein yellowing virus (CVYV) to discard possible infection mixture with one of the two most frequent whitefly transmitted virus of that growing area (Juárez et al. 2013). The other whitefly transmitted virus causing infections in Spanish cucurbit crops of the Mediterranean area, *Cucurbit yellow stunting disorder virus* (CYSDV), was not examined because it cannot be transmitted mechanically (Juárez et al. 2013; Navas-Castillo et al. 2014). Zucchini plants that tested positive for ToLCNDV and negative for CVYV were selected as virus source for mechanical inoculation experiments.

Mechanical inoculation method

In a first step, three different ToLCNDV inoculation procedures were tested for mechanical inoculation using seedlings of two genotypes which were highly susceptible to this virus under field conditions. The selected genotypes were one zucchini and one melon accession from Spain: MU-CU-16 (*Cucurbita pepo* subsp. *pepo*, morphotype Zucchini), and In-Ps-Piñ (*Cucumis melo* subsp. *melo* var. *inodorus* cv. Piñonet Piel de Sapo), both accessions maintained at the COMAV´s gene bank collection.

Ten seeds for each accession and inoculation method were disinfected by soaking them in 5 % sodium hypochlorite for 3 min. Subsequently, they were kept in Petri plates at 37 ^{o}C for 48 h to facilitate germination. Seedlings were transplanted to pots in a growth chamber under a photoperiod of 16 h day at 25 ^{o}C and 8 h night at 18 ^{o}C. For sap inoculation of ToLCNDV, 1 g of infected zucchini leaves was grounded with a mortar and a pestle in inoculation buffer. The resultant homogenate was used for inoculation of the two youngest fully expanded leaves of each plant, previously dusted with carborundum power 600 mesh, by gently rubbing with a cotton swab soaked with the crude homogenate. The three inoculums were prepared as follows (i) the buffer described in Sohrab et al. (2014) (0.1 M sodium phosphate [pH 7.2], 0.04 % 2-mercaptoethanol, 0.2 % sodium sulphite and 2 % celite) with a 1:4 proportion of infected leaves weight:buffer volume, (ii) the COMAV buffer (50 mM potassium phosphate [pH 8.0], 1 % polyvinylpyrrolidone 10, 1 % polyethylene glycol 6000, 10 mM 2-mercaptoethanol and 1 % activated charcoal) in a proportion of 1:4 (w:v), and (iii) COMAV buffer in a proportion of 1:10 (w:v). The procedure that resulted in a more efficient and severe infection in this preliminary assay was selected to inoculate ToLCNDV on different Cucurbitaceae taxa.

Inoculation of ToLCNDV on different Cucurbitaceae taxa

A total of 99 accessions from 33 different countries, representing four genera and 13 species (15 taxa if we consider subspecies level) were assayed to validate the efficiency of the selected mechanical inoculation method of ToLCNDV (Table 1). Seeds of most accessions were supplied by COMAV, USDA-NPGS and VIR genebanks and also a few accessions came from the Brazilian collection of UFERSA (Federal Rural University of the Semi-arid) and from the Indian collection of N. Dhillon (Dhillon et al. 2012). The number of plants tested per accession varied between 5 and 10 due to the lack of uniformity in the germination of the seeds. Accessions with all or most of the plants without symptoms at the end of assay were subjected to new inoculation rounds, using the same procedure, but with a higher number of plants to confirm the resistance.

Symptoms evaluation

In both assays (the preliminary assay to select the inoculation method and the screening assay), inoculated plants were kept in a growth chamber for ToLCNDV symptom evaluation for at least 30 days post-inoculation (DPI). One non-inoculated control plant per accession was kept separately to avoid virus infection. Symptoms were assessed by visual evaluation using an ordinal scale: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms or dead plant (Figure 1). The vulnerability index (V) was calculated for each taxon according to the following equation:

Vulnerability Index (V) = $\{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)/nt\ (nc-1)\}100$,

where, n_0, n_1, n_2, n_3, and n_4 are the number of plants with score 0, 1, 2, 3 and 4 respectively, nt is the total number of plants, and nc is the total number of categories (5).

This index has been used in previous screenings of *Luffa* spp against ToLCNDV (Islam et al. 2010; 2011) so its use allowed us a better comparison of our results with those of previous works. On the basis of this index and similarly to the classification

6

used by Islam et al. (2011), we considered resistant genotypes those with V = 0 − 25 %, moderately susceptible with V= 26 − 50 %, susceptible with V= 51 − 75 %, and highly susceptible V= 76 − 100 %.

ToLCNDV diagnosis by PCR

In all the assays ToLCNDV transmission was confirmed by PCR amplification and sequencing of random samples. Total DNA from apical leaves of inoculated and control plants was extracted two weeks after inoculation using the Cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). DNA was quantified and diluted with sterile distilled deionised water to a final concentration of 50 ng/µL. For ToLCNDV detection, 1 µL aliquots of total DNA (50 ng) were used as templates in PCR reactions of 20 µL with 1 U of Taq DNA polymerase (Biotools, Madrid, Spain), 1 µM primers To-1F (5´-GGGTTGTGAAGGCCCTTGTAAGGTGC-3´ position 476-501) and To-1R (5´-TGTACAGGCCATATACAACATTAATGC-3´ position 954-979), derived from the Spanish isolate Murcia 11.1 (segment DNA-A, accession number KF749225), and 0.2 mM dNTPs in buffer 75 mM Tris–HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO4$. The primer pair To-1F and To-1R was designed to amplify a specific region of the coat protein gene. Reactions were incubated for 5 min at 94 ^{o}C followed by 35 cycles of 30 s at 94 ^{o}C, 45 s at 55 ^{o}C and 45 s at 72 ^{o}C, with a final extension of 10 min at 72 ^{o}C. The resulting PCR products of 504 bp in length were analyzed by electrophoresis in 1 % agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate and 1 mM EDTA, pH 7.2) and stained with ethidium bromide.

To confirm the presence of ToLCNDV in the original inoculum and in random samples of the screening assays, amplified products were sequenced in both directions by means of an ABI PRISM DNA Sequencer 377 (Perkin–Elmer) by using To-1F and To-1R primers.

Results

Selection of mechanical inoculation method

Mechanical inoculation was successfully used to transmit ToLCNDV to zucchini squash (MU-CU-16) and Piel de Sapo melon (In-Ps-Piñ). Curling and mosaic were observed from 7-10 DPI and ToLCNDV infection was confirmed in the two genotypes by symptom scoring, specific PCR amplification with To-1F and To-1R primers and sequencing of the PCR product from random plants. The resulting sequence was 100% identical to the Spanish ToLCNDV isolates already published (Juárez et al. 2014). No ToLCNDV symptoms were observed in non-inoculated plants.

Differences in disease progression and symptoms severity were observed among the three inoculation procedures (Figure 2). A faster and more severe infection was found with the COMAV buffer in comparison with the buffer described by Sohrab et al. (2013; 2014). The highest percentage of systemic infection was obtained with the 1:4 ratio of leaves weight:buffer volume, that resulted in a 89 and 100 % of plants with severe symptoms in zucchini and melon, respectively, at 24 DPI, whereas the 1:4 w:v buffer of Sohrab et al. (2014) resulted in only 70 % of virus-positive plants, with mild to moderate symptoms, at this time. The infection efficiency was higher with the COMAV buffer at 1:4 w:v than at 1:10 w:v, with higher infection percentages, but similar symptoms in zucchini, and similar infection percentages, but more severe symptoms in melon (Figure 2). Therefore, the method described here using a 1:4 w:v ratio was shown to be very effective to mechanically inoculate ToLCNDV and was used for subsequent screening of different Cucurbitaceae taxa, in order to evaluate the potential seriousness of this virus on the main cultivated cucurbit species.

Inoculation of ToLCNDV on different Cucurbitaceae taxa

ToLCNDV was efficiently inoculated in the four assayed Cucurbitaceae genera: *Cucumis*, *Cucurbita*, *Citrullus* and *Lagenaria* (Table 1). The only accession screened of *L. siceraria* was highly susceptible (V = 80 %), displaying early severe symptoms (Figure 3A) and high viral load.

Several accessions of the other three genera were included in the assay. Virus accumulation and symptoms were quite variable, depending on the species considered and on the accession within the species, as described below.

Genus *Cucumis*

In general, melon accessions of *Cucumis melo* subsp. *melo* showed severe symptoms and high viral load (Table 1). The six accessions assayed of the *inodorus* and *cantalupensis* varieties (including sweet melons of most of the main market classes, such as Piel de sapo, Kirkagac, Charentais and Galia), and the six exotic accessions from Eastern Europe and Asia of the *ameri* and *flexuosus* varieties, were susceptible or highly susceptible to the mechanical transmission, with V > 55 % in *cantalupensis* and *flexuosus*, and V > 90 % in *inodorus* and *ameri* (Figure 3B). The only assayed accession of the *dudaim* variety behaved as moderately susceptible (V = 37 %).

ToLCNDV could be also mechanically transmitted to all the accessions of *C. melo* subsp *agrestis*, but symptoms were in general less severe that those found in subsp. *melo*, with differential results in the wide diversity of assayed varieties (Table 1). In fact, except from the only accession assayed of the *tibish* variety, that was susceptible (V = 70 %), the other varieties were classified as moderately susceptible or resistant (V from 23 to 48 %). In the six accessions of the Far Eastern *conomon* and the African and Indian *acidulus* varieties symptoms were delayed, but finally evolved to moderate/severe (Figure 3C), and were associated with medium to high viral load.

However, more erratic results were observed in Indian *momordica* accessions and in wild *agrestis* ones from India, Ghana, Senegal and Zimbabwe. In both groups, most accessions showed from mild to severe symptoms, but also asymptomatic accessions were found (Figure 3D-E). Accordingly, plants of some accessions were PCR negative or showed variable degrees of viral accumulation (Figure 4).

We also assayed three Spanish accessions of *C. sativus* that showed systemic infection and high viral load. Plants showed a delay in the development of symptoms and were less severe that those observed in *Cucumis melo* (Figure 3F). Finally, all plants from the only African accession of *Cucumis metuliferus* included in the assay were symptomless (Figure 3G) and ToLCNDV could not be detected by PCR in the apical non-inoculated leaves of any of these plants.

Genus *Cucurbita*

ToLCNDV also affected the three main cultivated species of the genus *Cucurbita*. In *C. pepo*, the most economically important species of the genus, mechanical

transmission was highly efficient (Table 1). *Cucurbita pepo* subsp. *pepo* morphotype Zucchini (Paris 2008) represents the main and more recent market class, and the five accessions were highly susceptible with V > 85 % (Figure 3H). First curly symptoms appeared around 7 DPI and became very severe around 15 DPI. Plant development was severely restricted and the incidence of the disease was so strong that a high percentage of the plants died before 20 DPI. A similar behavior (V > 90 %) was found in the 16 assayed accessions of the other three commercially important morphotypes of *Cucurbita pepo* subsp. *pepo*: Vegetable Marrow, Cocozelle and the ancient and more rustic Pumpkin (Figure 3I). The accessions of *C. pepo* subsp. *ovifera* (= *C. pepo* subsp. *texana* var. *ovifera*), which belonged to the morphotypes Acorn, Scallop and Crookneck, were highly susceptible to the virus, as well as the wild ancestor of the species, *C. pepo* subsp. *fraterna,* nearly all with V = 100 % (Table 1). The high values of the vulnerability index in all *C. pepo* accessions confirm the severity of this disease in this species.

Although economically less important than *C. pepo*, *C. maxima* and *C. moschata* are also worldwide cultivated. ToLCNDV was successfully transmitted to both species. In general, *C. maxima* developed a more severe infection (V = 57 % *vs* V = 32 %). Some *C. maxima* accessions had symptomless plants (Figure 3J), but most of them showed from moderate to severe symptoms (Figure 3K). Symptoms and viral accumulation were variable in *C. moschata*, with some accessions symptomless or with mild symptoms, with low or no viral accumulation (Table 1), and some other showing moderate to severe symptoms (Figure 3L-M).

ToLCNDV also caused severe infections in the assayed wild *Cucurbita* species. Some plants of *C. ecuadorensis* and *C. okeechobeensis* subsp. *martinezii* were severely affected (Figure 3N), although there was a high variability in symptoms severity and viral accumulation among plants. However, in *Cucurbita foetidissima* plants showed milder symptoms and lower virus accumulation (Figure 3O).

Genus *Citrullus*

The two accessions of *Citrullus lanatus* var. *lanatus* developed mild to moderate symptoms of curling and yellowing (Figure 3Q), with some plants being symptomless. They were classified as moderately susceptible (V = 38 %) (Table 1). Viral

accumulations were lower than those found in melons, pumpkins, and cucumbers, suggesting that this disease will be less harmful in watermelons. The only accession of *Citrullus lanatus* var. *citroides* included individuals with no or only mild symptoms (Figure 3P) and, accordingly, null or low virus load. Finally, all the plants of the only accession of *Citrullus colocynthis* included in the assay were symptomless and were PCR negative or showed quite low to moderate viral accumulation. Both the variety *citroides* and *C. colocynthis* were classified as resistant with V < 26 %.

Analysis of asymptomatic accessions of *C. melo* subsp. *agrestis*

As described above, we found three accessions of *Cucumis melo* subsp. *agrestis* var. *momordica* and two wild *C. melo* subsp. *agrestis*, all from India, in which most of the plants were asymptomatic and PCR-negative. To confirm the resistance of these accessions to the mechanical inoculation of ToLDCNV we repeated the inoculation assay with a higher number of plants and using In-Piñ-Ps as susceptible control. After three inoculation assays, all control Piel de sapo plants became systemically infected showing severe symptoms and high viral accumulation (V > 85 %), while all the plants of these accessions showed very mild symptoms, which disappeared as the plant develop, suggesting a infection recovery (average V ranging from 0 to 15%). Most of these plants had very low viral load or were found to be PCR-negative (Table 2).

Discussion

The mechanical transmission of ToLCNDV to individual plants provides an effective screening method to evaluate the level of resistance in a genotype that avoids the difficulties derived from whitefly management. Many begomoviruses have been reported to be mechanically transmissible to experimental hosts, such as *N. benthamiana*, but only some isolates of a few begomoviruses could be mechanically transmissible to other host plants. It has been speculated that differences between isolates in the length of the nuclear shuttle protein (NSP) may account for these differences. Mechanically transmissible isolates had shorter NSPs because of the loss of amino-acids in the N terminus, which has been shown to be decisive in symptom development and as a virulence determinant (Chang et al. 2010).

Only two isolates of ToLCNDV have been previously reported to be sap transmitted to some cucurbits. Chang et al. (2010) reported the mechanical transmission of a ToLCNDV isolate from Taiwan (ToLCNDV-OM) found in oriental melons (*Cucumis melo* var. *makuwa*, variety included in the group *conomon*) to the original host, to other accessions of the *conomon* group, and to four other cucurbits: bottle gourd, cucumber, zucchini squash, and sponge gourd. However, it could not be mechanically transmitted to watermelon, muskmelon (*Cucumis melo* var. *reticulatus,* variety included in the group *cantalupensis*), *Cucumis metuliferus* and *Cucurbita moschata.* Also an isolate from infected sponge gourds in India (ToLCNDV-[Luffa:Del) was transmitted to both sponge and ridge gourds (Sohrab et al. 2013; 2014), but failed to be transmitted to watermelon, muskmelon, cucumber, pumpkin and bottle gourd.

Other isolates have been shown to be transmitted to cucurbits by grafting and/or whitefly inoculations, without evidences of mechanical transmission. These included isolates from Thailandia (ToLCNDV-[Cuc:Tha]) transmitted to melons, cucumber and *Lagenaria leucantha* Rosby (Ito et al. 2008), India (ToLCNDV-IN[IN:ND]) transmitted to sponge, bottle and bitter gourds, cucumber and watermelon (Jyothsna et al. 2013), and Spain transmitted to melon, cucumber and squash (Juárez et al. 2014).

In the present study, the Spanish isolate (whose partial sequence is 100% identical to the one reported by Juárez et al. 2014) has been successfully sap transmitted to a wide host range. This includes most species that could not be infected with the other sap transmissible isolates, such as watermelon, muskmelon, *C. moschata* and species that were not tested in previous assays, such as *Cucurbita maxima* and many wild taxa related to the cultivated ones. It is remarkable that the Spanish isolate could not be transmitted to the only accession assayed of *Cucumis metuliferus*, similarly to the Asian isolates. Therefore, the Spanish isolate was transmitted to a host range wider than the two previously reported sap transmissible ToLCNDV isolates from Asia.

The transmission rate found in the two control accessions (*Cucurbita pepo* subspecies *pepo* morphotype Zucchini and *Cucumis melo* subsp. *melo* var. *inodorus* cv. Piel de sapo) was higher than 89 %, indicating that this is a cucurbit-infecting isolate with high mechanical transmission efficiency. The observed mechanical transmission rate is similar to that found with the two previously reported mechanically transmissible isolates in oriental melon (higher than 93 %) (Chang et al. 2010) and in ridge gourd

(higher than 80 %) (Sohrab et al. 2013). It is high enough to allow an accurate screening for resistance.

Sohrab et al. (2013) studied the factors that affect sap transmission of ToLCNDV in sponge gourd and developed a standardized protocol. However, with the Spanish isolate, the protocol developed by Sohrab et al (2013) did not resulted in high infection rates. Factors such as isolate, transmission buffer, inoculation procedure or host species may account for these differences.

Our results indicate that the Spanish isolate of ToLCNDV can be readily mechanically transmitted to the two most important crops of the genus *Cucumis*, melon and cucumber. Therefore, this virus is a potential serious problem in these crops, especially in melons of the subspecies *melo* in which we observed more severe infections in a wide diversity of germplasm, both commercial (such as cantalups, variety *cantalupensis*, and casaba melons, variety *inodorus*) and exotic resources originating from its diversification area (varieties *ameri*, *flexuosus* and *dudaim*). These exotic accessions are considered to be at the origin of commercial varieties and are an underutilized potential reservoir of interesting genes for breeding commercial melons (Pitrat 2008; Fernandez-Trujillo et al. 2011).

To date, most of the genes resistant to viruses used in melon breeding have been detected in genotypes belonging to two different varieties of the subspecies *agrestis*: *conomon* and *momordica* (Pitrat 2008). Delayed, but severe, symptoms associated to a high viral load were found in Far Eastern *conomon*. Similar results were observed in African and Indian *acidulus* and *tibish*. Interestingly we found some resistant accessions within the *momordica* variety and in wild *agrestis* from Northern India (Roy et al. 2012). Two of the *momordica* accessions, PI414723 and PI124112 have been previously reported to be resistant to many potyviruses, and were used to introgress these resistances in commercial melons as they are fully cross-compatible (Pitrat 2008; Dhillon et al. 2012). This is the first report of a source of resistance to ToLCNDV in melon, although this must be confirmed with whitefly transmission. The fact that this virus was firstly detected and spread in India, and that all the resistant accessions come from India, can also suggest a plant virus co-evolution in this area (Dhillon et al. 2012).

Cucumis metuliferus also seems to be resistant to the mechanical transmission of ToLCNDV, as previously reported by Chang et al. (2010) with the isolate ToLCNDV-OM. This species has been reported to be resistant to nematodes and soil fungal

pathogens, and has potential to be used as rootstock for cucumbers and melons (Álvarez et al. 2005; King et al. 2010; Munera et al. 2014). The lack of mechanical transmission may suggest a resistant behavior to this new virus. This is a valuable property that can facilitate its management in nurseries, but it needs to be demonstrated with new accessions and further inoculations using the transmission vector *B. tabaci*.

The results found in *Cucurbita pepo* suggest a more serious situation than that of melons. *Cucurbita pepo* subsp. *pepo* morphotype Zucchini (Paris 2008) represents the main and more recent market class, and all the accessions were highly susceptible. The other commercial morphotypes assayed, Vegetable marrow, Cocozelle and Pumpkin also displayed severe infections. The susceptibility of this species was much higher than that of the other cultivated species of the genus, especially *C. moschata* and *C. maxima*. Although they are economically less important than *C. pepo*, both are worldwide cultivated and have several diversification centers in different areas of the world, being staples in many developing countries (Ferriol and Picó 2008). They also have increasing importance since they have been selected as preferred rootstocks for other cucurbits (King et al. 2010). Results observed in *C. moschata* are promising, with some accessions asymptomatic and with a quite low viral load. This is one of the more rustic species within the genus *Cucurbita*. Some accessions of this species have been reported to be resistant to other viruses (Paris and Kabelka 2009). However, it remains to be demonstrated if this response is maintained after the inoculation with *B. tabaci*.

ToLCNDV also caused severe infections in most of the wild *Cucurbita* species, such as *C. ecuadorensis*, *C. okeechobeensis* and *C. foetidissima,* some of which have been reported to be resistant to other diseases (Paris and Brown 2005; Paris and Kabelka 2009). The fact that they are also highly susceptible to the disease confirm that this virus can be a serious threat to pumpkin and squash crops.

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Figure captions

Fig. 1 Symptom scoring in Piel de sapo melon plants (*Cucumis melo* subsp. *melo* var. *inodorus* cv. Piñonet Piel de Sapo (In-Ps-Piñ)) showing ToLCNDV symptoms corresponding to the scale: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms and 4, very severe symptoms or dead plant

Fig. 2 Effectiveness of three ToLCNDV inoculation methods on two highly susceptible accessions belonging to *Cucurbita pepo* subsp. *pepo* morphotype Zucchini (MU-CU-16) and *Cucumis melo* subsp. *melo* var. *inodorus* cv. Piñonet Piel de Sapo (In-Ps-Piñ). The solid line represents the method performed by Sohrab et al. (2014); the dotted line represents the method described here using a 1:10 ratio of leaves weight:buffer volume; and the dashed line represents the same method with a 1:4 ratio. Percentage of virus-positive plants is given at 12, 16 and 24 days post inoculation. Averaged symptoms were evaluated on a scale from 0 (no symptoms) to 4 (very severe symptoms)

Fig. 3 Symptoms exhibited after mechanical inoculation of ToLDCNV in different species, subspecies and varieties from genera *Lagenaria, Cucumis, Cucurbita* and *Citrullus.* **A,** *Lagenaria siceraria* BGV-217. **B,** *Cucumis melo* subsp. *melo* var. *cantalupensis* cv. *Vedrantais,* Charentais market class. **C,** *Cucumis melo* subsp. *agrestis* var. *makuwa* cv. *Ginsen makuwa.* **D,** *Cucumis melo* subsp. *melo* var. *momordica* PI-124112. **E,** *Cucumis melo* subsp. *agrestis* var. *agrestis* WM9. **F,** *Cucumis sativus* AN-C-158. **G,** *Cucumis metuliferus* BGV-11135. **H,** *Cucurbita pepo* subsp. *pepo* Zuchinni MU-CU-16. **I,** *Cucurbita pepo* subsp. *pepo* Pumpkin PI-171628. **J,** *Cucurbita maxima* VAV-1860. **K,** *Cucurbita maxima* VAV-3202. **L,** *Cucurbita moschata* Nigerian local. **M,** *Cucurbita moschata* PI-482527. **N,** *Cucurbita ecuadorensis* PI-4342443. **O,** *Cucurbita foetidissima* PI-442201. **P,** *Citrullus lanatus* var. *citroides* BGV-5167. **Q,** *Citrullus lanatus* var. *lanatus* BGV-65. Seeds of accessions were supplied by USDA-NPGS (PI), COMAV (BGV-MU-AN) and VIR (VAV) genebanks and by the collection of Narinder Dhillon (WM) (Dhillon et al. 2012)

Fig. 4 Polymerase chain reaction detection of ToLCNDV in plants classified as resistant from different *Cucumis melo* subsp. *agrestis* accessions. Lane 1, the highly susceptible

genotype of *C. melo* subsp. *melo* var. *inodorus* cv. Piñonet Piel de Sapo showing high viral accumulation (+++); lanes 2-4 asymptomatic accessions from genotypes *C. melo* subsp. *agrestis* var. *momordica* showing intermediate (++, lane 2), low (+, lane 3) and null (-, lane 4) viral accumulation. C^+ and C^-, positive and negative controls from the original source of inoculum of ToLCNDV and non-inoculated zucchini plants, respectively. The templates used were 50 ng of total DNA from apical non-inoculated leaves. M, molecular size marker

Table 1. Response of the different accessions used in the screening assay to mechanical inoculation with the Spanish isolate of ToLCNDV.

Genus	Species[a]	Subspecies[a]	Morphotype or variety	Number of accessions	Origin	V (in %)[b] / Viral load[c]
			Zucchini	5	Algeria, Morocco, Spain	86.72 / +++
		pepo	Pumpkin	5	Spain, Turkey	92.50 / +++
			Veg. Marrow	6	Morocco, Spain	92.50 / +++
	pepo		Cocozelle	5	Italy, Greece, Spain	97.83 / ++ to +++
			Scallop	1	Spain	100 / +++
		ovifera	Crookneck	1	USA	100 / +++
Cucurbita			Acorn	2	USA	97.50 / +++
	fraterna			1	Mexico	100 / +++
	moschata			12	China, Cuba, Costa Rica, India, Japan, Nigeria, Spain, USA, Zimbabwe	32.27 / - to +++
	maxima			8	Angola, Argentina, Australia, Chile, Morocco, Peru, Spain	57.05 / - to +++
	ecuadorensis			1	Ecuador	100 / - to +++
	okeechobeensis	*martinezii*		2	Mexico	20.00 / - to +++
	foetidissima			2	Mexico	12.50 / - to +
			inodorus	4	Spain, Tunisia, Turkey	90.97 / +++
			ameri	3	Iran, Russia, Turkey	93.75 / +++
		melo	*cantalupensis*	2	Libia, France	69.23 / +++
			dudaim	1	Afghanistan	37.30 / ++
	melo		*flexuosus*	3	India, Irak	55.00 / +++
			conomon	4	Japan, Korea	47.66 / + to +++
Cucumis			*momordica*	10	Brazil, India	29.58 / - to +++
		agrestis	*acidulus*	2	India, Zimbabwe	32.50 / ++ to +++
			tibish	1	Sudan	70.00 / +++
			wild type	9	Ghana, India, Senegal, Zimbabwe	22.73 / - to +++
	sativus			3	Spain	50.00 / +++
	metuliferus			1	Africa	0.00 / -
Lagenaria	*siceraria*			1	Morocco	80.00 / +++
	colocynthis			1	Spain	0 / - to ++
Citrullus			*lanatus*	2	Spain	37.50 / - to +
	lanatus		*citroides*	1	Spain	25.00 / - to ++

[a] *Cucurbita pepo* L. ssp. *pepo*, *Cucurbita pepo* L. ssp. *ovifera* (L.) D.S. Decker (= *texana* var *ovifera*), *Cucurbita fraterna* L.H. Bailey, *Cucurbita moschata* Duchesne, *Cucurbita maxima* Duchesne, *Cucurbita ecuadorensis* H.C. Cutler & Whitaker, *Cucurbita okeechobeensis* L.H. Bailey ssp. *martinezii* (L.H. Bailey) T.W. Walters & D.S. Decker, *Cucurbita foetidissima* Kunth, *Cucumis melo* L. ssp. *melo*, *Cucumis melo* L. ssp. *agrestis* (Naudin) Greb., *Cucumis sativus* L., *Cucumis metuliferus* E. Mey. ex Schrad., *Lagenaria siceraria* Standl., *Citrullus colocynthis* (L.) Schrad., *Citrullus lanatus* (Thunb.) Matsum. & Nakai.

[b] V: Vulnerability index at 30 days post inoculation.

[c] See Figure 4 for the scale.

Table 2. Response of five resistant accessions of *C. melo* subsp. *agrestis* (variety *momordica* and wild *agrestis*) to mechanical inoculation with the Spanish isolate of ToLCNDV. Seeds of accessions were supplied by USDA-NPGS (PI), COMAV (Kharbuja) and by the collection of N. Dhillon (WM) (Dhillon et al. 2012).

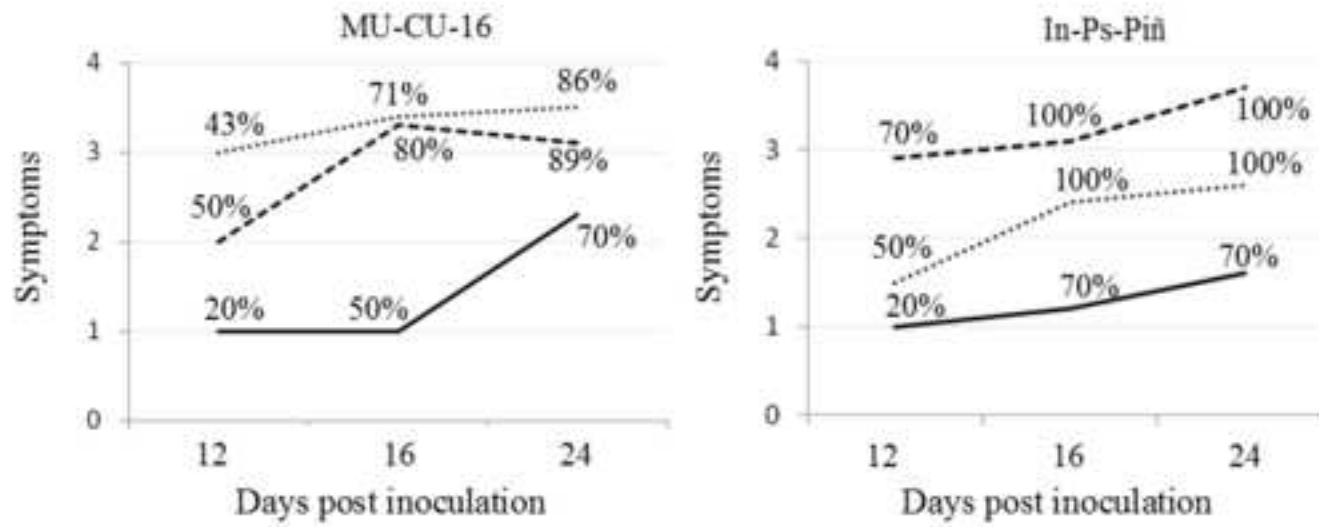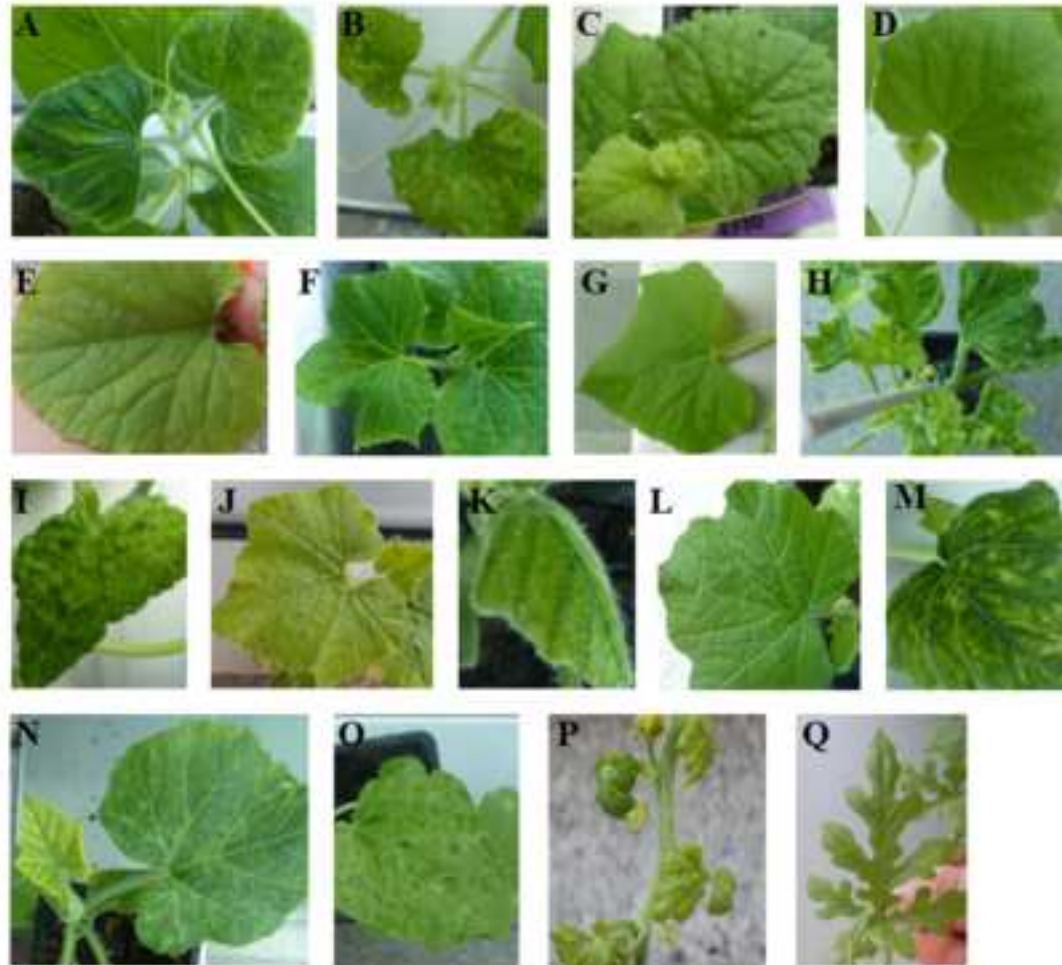| *Cucumis melo* subsp. *agrestis* var. *momordica*			
Accession (Code/Name)	V (%) at15 DPI Assay1/2/3 Average ± Standard deviation	V (%) at 30 DPI Assay1/2/3 Average ± Standard deviation	Virus load
Mom-KhaInd/ Kharbuja	20/10/12.5 14.2±5.2	30/15/0 15±15	- to ++
Mom-PI124Ind/ PI 124112	15/10/6.3 10.4±4.4	0/10/0 3.3±5.8	- to +
Mom-PI124Ind/ PI 414723	10/0/2.8 4.3±5.2	0/0/0 00±0	- to ++
Cucumis melo subsp. *agrestis* wild types			
Ag-WM9Ind/WM9	25/7.1 16.1±12.7	0/0/0 00±0	- to -/+
Ag-WM7Ind/WM7	5/0 1.7±2.9	0/0/0 00±0	- to -/+
Cucumis melo subsp. *melo* var *inodorus*			
In-Piñ-Ps/Piñonet Piel de sapo(control)	68.8/60/75 59.7±17.6	100/100//75 85.4±12.6	+++
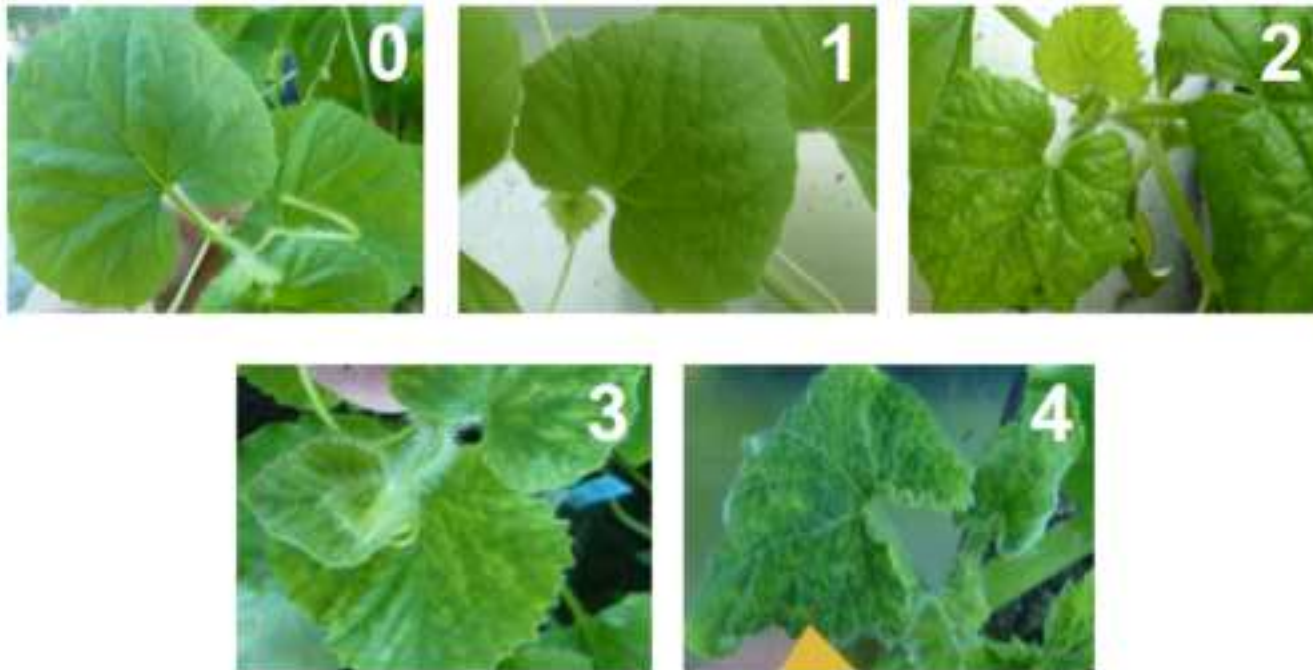
Figure 1

Figure 2

Figure 3

Figure 4