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Resistance to *Tomato leaf curl New Delhi virus* (ToLCNDV) in *Cucurbita* spp.

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Abstract

Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus (family *Geminiviridae*) first reported in India and neighboring countries. ToLCNDV severely affects zucchini crop (*Cucurbita pepo*) in the main production areas of Southern Spain since 2012. This emerging begomovirus is a serious threat to this and other cucurbit crops. Breeding resistant cultivars is the most promising method for disease control, but requires the identification of sources of resistance in the *Cucurbita* genus. In this work, we screened for ToLCNDV resistance a large collection of *Cucurbita* spp. accessions, including landraces and commercial cultivars of the main cultivated species, *C. pepo*, *C. moschata* and *C. maxima,* and wild species. The screening was performed using mechanical and whitefly inoculation. The level of resistance was assessed by scoring

symptom severity and by measuring the virus content with quantitative PCR in selected genotypes. Diversity in the response was observed within and among species. Severe symptoms and high viral amounts were found at 30 days after mechanical and whitefly inoculation in *C. pepo*, in all accessions belonging to the Zucchini morphotype and to other morphotypes of both subspecies, *pepo* and *ovifera,* and even in the wild relative *C. fraterna*. *Cucurbita maxima* was also highly susceptible. This species showed characteristic symptoms of leaf decay and intense yellowing, different from those of mosaic, curling, and internode shortening found in *C. pepo*. The only species showing resistance was *C. moschata*. Four accessions were symptomless or had some plants with only mild symptoms after three independent rounds of mechanical inoculation with different inoculum sources. Two of them also remained symptomless after virus inoculation with viruliferorus whiteflies. ToLCNDV was detected in these asymptomatic accessions at 15 and 30 dpi, but viral amounts were much lower than those found in susceptible genotypes, suggesting a high level of resistance. The symptoms in the susceptible accessions of this species were also different, with a characteristic leaf mottling, evolving to a severe mosaic. The newly identified *C. moschata* resistant accessions are good candidates for breeding programs to avoid the damage caused by ToLCNDV.

Introduction

Tomato leaf curl New Delhi virus (ToLCNDV) is a member of the genus *Begomovirus* (family *Geminiviridae*), with a bipartite genome, comprised of two circular single-stranded DNA molecules of approximately 2.7 kb each (designated as DNA-A and DNA-B), which are encapsidated in geminate particles. Both DNA-A and DNA-B encoded transcripts are required for infection and symptom development in host plants, although the DNA-A component is capable of autonomous replication inside the host (Papidam *et al*., 1995; Fauquet *et al*., 2008; Ito *et al*., 2008). ToLCNDV is transmitted in a persistent manner by the whiteflies of the *Bemisia tabaci* sibling species group (Chang *et al*. 2010; Islam *et al*., 2010; Khan *et al*., 2012; Jyothsna *et al*., 2013).

ToLCNDV was first reported on tomato (*Solanum lycopersicum* L.) in India (Srivastava *et al*., 1995; Papidam *et al.,* 1995). Later, it was found in neighboring countries on several hosts, particularly vegetable species of the Cucurbitaceae and Solanaceae families (Chang *et al*., 2010; Pratap *et al*., 2011; Khan *et al*., 2012; Jyothsna

et al., 2013; Bandaranayake *et al*., 2014). During the last decade, its host range has increased and the virus has invaded new countries, arriving into Europe. A severe outbreak of ToLCNDV occurred in greenhouse and field-grown zucchini and melon crops in the main production area of Southern Spain in 2012-2013 (Juárez *et al*., 2014). Since then, this virus has been causing a great impact with catastrophic losses in this horticultural region, and is considered a serious threat to these and other cucurbit crops in the Mediterranean area.

Spain is one of the main world producer of zucchini and melon (FAOSTAT, 2015), and the first exporting country in Europe. The production of these crops has been severely affected by a number of viruses, particularly RNA viruses transmitted by aphids (Ferriol and Picó, 2008; Paris, 2008). However, apart from the typical New World begomovirus *Squash leaf curl virus* (SLCV), begomovirus association with zucchini and melon has been so far unknown in this region (Lecoq & Desbiez, 2012).

In many regions of the world, control strategies for begomovirus diseases focus on vector management. Several approaches including insecticide applications and physical barriers are used for reducing establishment of whitefly populations. In addition, cultural practices such as virus-free transplants, crop-free periods, weed management and rouging of infected plants are suggested for managing whiteflies (Seal *et al*., 2006; Lecoq & Desbiez, 2012; Janssen *et al*., 2014). However, these vector management strategies are not always fully effective. The complex epidemiological factors associated with these diseases, such as broad host range, accelerated rates of virus and vector evolution and the migratory behaviour of whiteflies hinder the development of effective long-term management strategies (Snehi *et al*., 2015). Therefore, breeding resistant cultivars is an essential element of a sustainable approach to manage the diseases caused by begomoviruses.

Since ToLCNDV was first detected affecting tomato and other solanaceous crops (Naqvi *et al*., 2010; Sahu *et al*., 2012; Rai *et al*., 2013; Ruíz *et al*., 2015), resistance studies are more advanced in this family (Kushwaha *et al*., 2015), and the search for resistance in cucurbits has not been a primary goal in the affected countries. Nonetheless, resistance screenings have been reported in sponge gourd (*Luffa cylindrica* M. Roem.), a popular cucurbit vegetable in India severely affected by this virus (Islam *et al.,* 2010, 2011). Although most ToLCNDV isolates are naturally transmitted only by whiteflies, some of them have been shown to be mechanically (sap) transmitted to different hosts (Samretwanich *et al*., 2000; Usharani *et al*., 2004; Chang *et al*., 2010;

Sohrab *et al*., 2013), including the new Spanish isolates. In a previous work, we developed a protocol for mechanical inoculation using a ToLCNDV isolate from Almeria, in southern Spain. Using this protocol, we demonstrated that this isolate has a wide host range, as it was successfully transmitted to four genera and 13 species of the Cucurbitaceae family, including the main crop species, such as cucumber, watermelon, melon, squash and zucchini, as well as crop-related exotic germplasm. The availability of this highly efficient method for mechanical transmission facilitated the identification of resistance in Indian melons (López *et al*., 2015). This resistance is now being used to develop resistant melon cultivars.

There is an urgent need of developing resistant cultivars in zucchini. Losses in this crop are being especially devastating (Alfaro & Font, 2014; Janssen *et al*., 2014). The mechanical transmission method developed by López *et al*. (2015) also allowed a preliminary study of the response of the main species of the genus *Cucurbita*. In general, the susceptibility of *C. pepo* L. was much higher than that of the other cultivated species of the genus, especially *C. moschata* Duchesne and *C. maxima* Duchesne. This preliminary assay suggested a differential response of the species in the genus that needs to be further characterized and confirmed under natural infection with the vector to be useful in the development of resistant cultivars. Here we report the screening of a collection of 110 *Cucurbita* accessions selected to represent the variability in the genus with both mechanical transmission, using different virus sources, and whitely inoculation. The identification of two *C. moschata* accessions highly resistant to both mechanical and whitefly inoculation, which remained symptomless and showed a reduced viral accumulation, provides the first sources for breeding ToLCNDV-resistant *Cucurbita* cultivars.

Materials and methods

Plant material

A total of 110 *Cucurbita* accessions were first screened in two assays, one in climatic chamber using mechanical inoculation with a ToLCNDV isolate from affected fields in Almeria, and the second during spring-summer season in Almeria under greenhouse conditions with viruliferous whiteflies (Tables 1 and 2). The *Cucurbita* collection represents the three main cultivated species of the genus, *C. pepo* [64 accessions of subsp. *pepo* and nine of subsp. *ovifera* (L.) D.S. Decker (= *texana* var. *ovifera*), and two

F1 hybrids (subsp. *pepo* x *pepo* and subsp. *pepo* x *ovifera*)], *C. maxima* (14), and *C. moschata* (14), as well as six accessions of four wild types (two of *C. fraterna* L.H. Bailey, two of *C. okeechobeensis* L.H. Bailey subsp. *martinezii* (L.H. Bailey) T.W. Walters & D.S. Decker, one of *C. lundelliana* L.H. Bailey, and one of *C. foetidissima* Kunth), and one of the cultivated *C. ficifolia* Bouché. All these accessions were selected from a collection of around 600 entries maintained at the germplasm bank of the Institute for the Conservation and Breeding of Agricultural Biodiversity (COMAV). Some of them were collected by the COMAV team and others originated from exchanges with other germplasm banks (mainly USDA-NPGS and CATIE). This selection aimed to represent the variability of the full collection. In *C. pepo* the two subspecies (*pepo* and *ovifera*) and the main morphotypes within each subspecies (subsp. *pepo*: Pumpkin, Vegetable Marrow, Zucchini, Cocozelle; subsp. *ovifera*: Acorn, Scallop, Croockneck; and ornamental gourds) were represented. In *C. maxima* and *C. moschata*, accessions from the center of origin and from secondary centers of diversity were included.

A selection of accessions having all or most of the plants with no or mild symptoms at the end of both screening assays (mechanical and whitefly inoculation) was assayed again to confirm their response. In this second experiment we used mechanical inoculation in a climatic chamber with two inocula (the same inoculum from infected fields used previously and a new one obtained from an infectious clone as described below) to confirm the resistance of the selected accessions and to validate the use of the infectious clone in resistance screenings.

Virus sources for mechanical inoculation

ToLCNDV infected zucchini plants from Almeria were the original source of inoculum for mechanical inoculation as described in López *et al*. (2015). The virus was transmitted to zucchini seedlings of the susceptible accession MU-CU-16 by virus-free whiteflies. Leaf extracts from these zucchini plants were collected fifteen days after whitefly transmission and used as virus source for the first screening assay with the whole *Cucurbita* collection and for the second assay to confirm the response of some selected genotypes.

In this second assay, an additional virus source was used in mechanical transmissions, derived from a ToLCNDV infectious clone. Dimeric clones of the DNA-A and DNA-B of a ToLCNDV isolate, from an infected zucchini plant in Almeria, were

generated using rolling circle amplification (RCA) and cloned into the binary vector pBINPLUS (Engelen *et al*., 1995). The clones were fully sequenced and showed 99% nucleotide identity with the sequence of the Spanish ToLCNDV isolate (KF749224 and KF749225; Juárez *et al*., 2014). Clones pBIN2TOA4R and pBIN2TOB14R with the complete dimers for DNA-A and DNA-B, respectively, were used separately for the transformation of *Agrobacterium tumefaciens* LBA4404. Two cultures of *A. tumefaciens,* each transformed with infectious clones pBIN2TOA4R or pBIN2TOB14R and grown in the selective media containing 25 μg mL^{-1} rifampicin and 50 μg mL^{-1} kanamycin, were sedimented by centrifugation, adjusted to 0.5 OD_{600}, induced for 2 h at 28°C and infiltrated by injection into petioles of MU-CU-16 zucchini plants. Fifteen days after agroinoculation, leaf extracts from plants showing ToLCNDV symptoms were used as virus source for mechanical inoculation in the second screening assay performed to confirm the response of some selected accessions.

Mechanical inoculation

With either inoculum from field or derived from the infectious clone, mechanical inoculation of ToLCNDV was performed as described in López *et al*. (2015). Briefly, 1 g of infected zucchini leaf tissue was ground in inoculation buffer in a 1:4 (w:v) proportion. The resultant homogenate was used for inoculation of one cotyledon and one fully expanded leaf of each plant, previously dusted with carborundum (600 mesh), by gently rubbing with cotton-bud sticks soaked with the crude homogenate.

For the mechanical inoculation, ten plants per accession were inoculated in the first screening with the whole *Cucurbita* collection, and five plants per accession were inoculated with each of the two *inocula* (field and infectious clone) in the second assay performed to confirm the response of some selected accessions. In both assays, two additional plants per accession were mok-inoculated with buffer and carborundum, or not inoculated to be used as negative controls. Seeds were disinfected by soaking them in 5% sodium hypochlorite for 3 min. Subsequently, they were kept in Petri dishes at 37°C for 48 h and seedlings were transplanted to pots in a climatic chamber with controlled environmental conditions of 25°C/18°C day/night temperature, 60/95% day/night relative humidity, and a 16–8 h light/dark photoperiod. Seedlings at the three true-leaf stage were mechanically inoculated.

Whitefly inoculation

Seedlings at the three-four true leaf stage of the *C. pepo* susceptible cultivar Sinatra were transplanted into the greenhouse on 15 March. Infected adult plants of the same cultivar with clear symptoms of ToLCNDV, coming from an infected field in Almeria, were transplanted 37 days later to establish a population of viruliferous whiteflies in the greenhouse. PCR analysis was used, before transplanting, to confirm that the adult plants contained ToLCNDV, but not CVYV (*Cucumber vein yellowing virus)* (Picó *et al*., 2005) or CYSDV (*Cucumber yellowing stunting disorder virus*), two other local viruses transmitted by whiteflies. Once whiteflies were established in the plants of the first transplanting and these started to show symptoms of ToLCNDV, seedlings of the different *Cucurbita* accessions to be evaluated were distributed in the greenhouse and kept in nursery trays till the end of the assay. Two replications of six plants each were evaluated for each accession. For the first replication, the infection started on 30 April and for the second on 13 May. In both cases the assay was concluded 35 days later.

Symptoms evaluation and virus detection by PCR

In the first screening with the whole *Cucurbita* collection using mechanical inoculation, plants were kept in a climatic chamber and every plant was evaluated for ToLCNDV symptoms at 30 dpi (days post inoculation). Symptoms were assessed visually, using a scale from 0 (absence of symptoms) to four (very severe symptoms or dead plant) detailed in López *et al*. (2015). The same conditions were used in the second assay, performed to confirm the response of some selected accessions, but symptoms were scored at 15 and 30 dpi. In this assay the presence of the virus was analyzed at 15 and 30 dpi using a PCR reaction designed to detect the presence of both viral components. Total DNA from apical leaves was extracted using the CTAB method (Doyle & Doyle, 1990). DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted to a final concentration of 50 ng μL^{-1}. One µL aliquots of total DNA (50 ng) were used as templates in PCR reactions of 25 µL, containing 1 U of Taq DNA polymerase (Biotools, Madrid, Spain), 1 µM of two different primer pairs (To-A1F/To-A1R, and To-B1F/To-B1R) and 0.2 mM dNTPs in 75 mM Tris–HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl and 20 mM $(NH4)_2SO_4$. The two primer pairs were derived from the Spanish isolate Murcia 11.1, one from the DNA-A, accession number KF749225, (To-A1F 5´-GGGTTGTGAAGGCCCTTGTAAGGTGC-3´, positions 476-501, and To-A1R 5´-AGTACAGGCCATATACAACATTAATGC-3,´ positions 954-979), and the other from the DNA-B, accession number KF749228, (To-

B1F 5′-GAAACACAAGAGGGCTCGGA-3′, positions 637-656, and To-B1R 5′-GCTCCACTATCAAAGGGCGT-3′, positions 1294-1313). Cycling conditions consisted of incubation at 94°C for 5 min and 45 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s, with a final extension of 10 min at 72°C. The resulting PCR products of 504 and 677 bp in length were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer.

A quantitative polymerase chain reaction (qPCR) assay was also performed in selected samples to estimate virus titer in the most resistant accessions. Three biological samples (plants per genotype) were analyzed at 15 and 30 dpi. Amplifications were done with primers designed from the DNA-A: ToLCNDVF1 (5′-AATGCCGACTACACCAAGCA-3′, positions 1145-1169) and ToLCNDVR1 (5′-GGATCGAGAAGAGAGTGGCG-3′, positions 1399-1418), producing a fragment of 274 bp. The qPCR was performed in a Rotorgene thermocycler (Qiagen, Hilden, Germany). The reaction mix contained 7.5 µL of iTaq Universal SYBR Green supermix (2×) (BIORAD, Hercules, United States), 1 µM of each primer and 1.5 µL of total DNA. Cycling conditions consisted of incubation at 95°C for 5 min and 40 cycles of 95°C for 5 s and 60°C for 30 s. Three technical replications were performed per sample. Relative accumulation of ToLCNDV in the plants was calculated by the comparative Ct (Cycle Threshold) method, using the gene CpACS27A from *C. pepo* as an internal standard (ACS27FWDRACE 5′-CCACTTGGTGCCACAATCCAACGG-3′, and ACS27REVRACE 5′-GCCTATCCAAAGACCTCGGCCTTCCC-3′). Firstly we demonstrated that the efficiency of amplification for each amplicon was roughly equivalent, regardless of the amount of template cDNA. The relative accumulation of the virus to a calibrator sample was calculated using the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference between the ΔCt of each sample and the ΔCt of the calibrator sample.

Symptoms after whitefly transmission in the greenhouse assay with the whole *Cucurbita* collection were scored using the same scale, at 21, 28, and 35 dpi (days after the introduction of plants in the greenhouse with the viruliferous whitefly population). qPCR was also used to estimate virus titer in a selected set of the accessions analyzed in the greenhouse. Two biological replications (two pools of six plants) were analyzed per genotype at 28 dpi, using the same procedure described above.

Data analysis

Resistance was evaluated as the host response to virus infection estimated from symptom severity and in some selected genotypes from viral titre. In the first screening assay, the percentage of symptomatic plants and the mean and range of symptom scores were calculated for each genotype after mechanical and whitefly inoculation. The mean and range of symptom scores were also calculated, along with the percentage of PCR positive plants, in the second assay performed to confirm resistance of selected genotypes. The viral titer was estimated by qPCR in some selected plants representing different responses.

Results

Response of *Cucurbita* spp to mechanical and whitefly-mediated ToLCNDV transmission

A total of 75 accessions of *C. pepo* were assayed. Most of them were highly susceptible to the mechanical transmission of ToLCNDV, developing severe to very severe symptoms at 30 dpi (mean symptom score 3.6, ranging from 2.3 to 4) (Table 1). The observed symptoms included upward and downward curling and severe mosaic of young leaves and short internodes (Figure 1A). Natural infection revealed differences in infection progress among the *C. pepo* accessions at 21 dpi (Table 1). However, all of them had very severe symptoms at the end of the assay (mean symptom score of 1.2 and 3.9 at 21 and 35 dpi respectively).

The accessions assayed represented the main morphotypes of the two subspecies of *C. pepo* (*pepo* and *ovifera*), and the response to mechanical transmission of ToLCNDV was similar in all of them (Table 1). All the accessions of the Pumpkin and Vegetable Marrow morphotypes, from diverse origins, were highly susceptible (mean symptom score 3.5 and 3.7, respectively, at 30 dpi). The Spanish and Italian accessions of the two more modern morphotypes of subsp. *pepo*, Zucchini and Cocozelle, were also found to be highly susceptible (mean symptom score 3.4 and 3.5, respectively). Results indicated that accessions of the subspecies *ovifera*, both edible and ornamental types, were as susceptible as those of subsp. *pepo* (mean symptom score 3.8). The two Mexican accessions of the wild species *C. fraterna* were as highly susceptible to ToLCNDV as the cultivated genotypes (mean symptom score 3.8). Finally, the two F1 hybrids (subsp. *pepo* x *pepo* and subsp. *pepo* × *ovifera*) assayed as a part of a breeding program for developing new *Cucurbita* rootstocks were also susceptible. The early response to

whitefly inoculation was less severe in wild *C. fraterna* (Figure 2). Differences in plant vigour may partly account for these differences as the less vigorous genotypes, belonging to wild *C. fraterna*, might have a delayed expression of virus symptoms (Table 1). However, in all cases a very severe infection was observed at the end of the greenhouse assay.

Cucurbita maxima accessions, mainly from America and Africa, were susceptible to ToLCNDV (mean symptom score 3.1, ranging from 2.2 to 4), in general with less severe symptoms than the *C. pepo* accessions (Table 2). However, although whitefly inoculation caused a delayed and less severe infection at the beginning of the assay in *C. maxima* in comparison with *C. pepo*, at the end of the assay both species gave similar results with 100% symptomatic plants and very severe symptoms (Figure 2). This species showed characteristic symptoms of leaf decay and intense yellowing, different from those found in *C. pepo* (Figure 1B).

The only accession assayed of *C. ficifolia* was highly susceptible to the infection (mean symptom score 4). The wild species assayed showed variable responses to mechanical inoculation (mean symptom scores from 0 to 4) (Table 2), but were all highly susceptible after whitefly inoculation.

Cucurbita moschata showed less severe symptoms than the other species (Table 2). Most of the accessions assayed had mean scores of symptoms from mild to moderate after mechanical inoculation (mean symptom score 1.7, ranging from 0.2 to 4). Whitefly inoculation resulted in a significantly delayed infection with variable symptoms, from mild to very severe symptoms at the end of the assay (Figure 2). Susceptible accessions developed severe symptoms with characteristic leaf mottling evolving to severe mosaic, but without the leaf curling and the internodes shortening found in *C. pepo* (Figure 1C). Four accessions displayed interesting results after mechanical inoculation: PI 604506 (the cultivar Cheese Large) from the USA, PI 381814 from India, Nigerian Local from Nigeria, and Kurokawa from Japan. All had mean symptom scores below 1, with all plants ranging from mild to no symptoms. The Indian and American accessions (PI 381814 and PI 604506) also remained symptomless after whitefly inoculation, whereas Nigerian Local and Kurokawa showed some plants with severe symptoms under greenhouse conditions (Table 2).

The accumulation of ToLCNDV was evaluated in two pools, each of six plants, in nine selected *C. moschata* genotypes, representing a range of responses after whitefly inoculation (Figure 3). These were the four accessions having scores below 1 after the

mechanical inoculation (PI 604506, PI 381814, Nigerian Local and Kurokawa), two additional accessions that remained with moderate symptoms after both mechanical and whitefly inoculation (PI 550689 and AN-CU-45), and three accessions that had moderate symptoms after mechanical inoculation, but severe at the end of the whitefly assay (PI 264551, IVIA 205 and PI 369346). Five highly susceptible accessions, three *C. pepo* and two *C. maxima,* were used as controls. The *C. pepo* and *C. maxima* susceptible controls showed the highest accumulation of the virus, which was similar in both species (Figure 3). ToLCNDV was also detected in the *C. moschata* accessions. The accessions with severe symptoms at the end of the assay had viral titres between 15 and 1.5 times lower than the other species. *Cucurbita moschata* accessions showing no or mild to moderate symptoms (PI 604506, PI 381814, Nigerian Local, Kurokawa and AN-CU-45) displayed the lowest viral titres.

Confirmation of ToLCNDV resistance in *C. moschata*

The response of the resistant *C. moschata* genotypes was confirmed in a second screening assay, along with some selected *C. pepo* and *C. maxima* accessions that showed different symptom levels in the first assay. Both the inoculum derived from zucchini field-infected plants used before and the new one coming from the ToLCNDV infectious clone were used. Results of these inoculations are shown in Table 3, and confirmed the previously obtained results. All accessions showed similar results and no differences were found in the evolution of symptoms between the two inocula sources, thus confirming the utility of the infectious clone for resistance screenings.

All the assayed plants were sampled for ToLCNDV detection by PCR. Both DNA components were detected in most plants of all genotypes, confirming the viral infection, even in symptomless plants (Table 3). Highly severe infections were confirmed in *C. pepo, C. fraterna* and *C. ficifolia*. The highly susceptible plants of these species had symptoms evolving from moderate to severe. Similarly to the first screening assay, symptoms were initially less severe in the selected *C. maxima* accessions, but evolved to very severe in most accessions. A moderate infection was found in *C. lundelliana* and *C. ockeechobensis*, with lower symptom scores than in *C. foetidissima.* The four accessions of *C. moschata* selected previously remained with symptom scores below 1.0 after the two independent inoculations. ToLCNDV was detected in plants of these accessions. Positive results with standard PCR indicated that ToLCNDV is present even in the symptomless accessions. qPCR performed with four selected

accessions gave similar results to those obtained after whitefly inoculation. The two resistant *C. moschata* (PI 604506, PI 381814) had very low viral titers, and the susceptible *C. moschata* accession PI 482527 had viral load five times lower than that found in the susceptible accession of *C. pepo* used as control.

Discussion

Begomoviruses had not been a main problem of cucurbits in Europe until recently (Lecoq and Desbiez, 2012). However, the increasing and severe impact of *Tomato leaf curl New Delhi virus* in Zucchini fields in Southern Spain (Alfaro & Font, 2014; Janssen *et al*., 2014) points to this virus as the most serious threat of this crop in the Mediterranean region, the main suppliers of vegetables to Europe.

Our results confirm the high susceptibility of the species *C. pepo* to both mechanical and whitefly transmission of ToLCNDV. The knowledge of the genetic diversity of the species (Formisano *et al*., 2012; Esteras *et al*., 2013; Gong *et al*., 2012) allowed us to select a set of accessions representing most of the main morphotypes of the two subspecies of *C. pepo*. These accessions included the most ancient and rustic morphotype of the subsp. *pepo*, the Pumpkin morphotype (Paris *et al*., 2003; Ferriol *et al*., 2003; Ferriol *et al*., 2007), but also landraces belonging to the Vegetable Marrow morphotype, developed in Europe after European contact with America and still appreciated in Mediterranean countries (Paris & Brown, 2005), and the more modern Cocozelle and Zucchini morphotypes, developed in Italy in the last century (Gong *et al.*, 2012). Despite the diversity of the collection, no total or partial resistance or useful tolerance were identified in this subspecies. Similar susceptibility was found in the American accessions representative of the *ovifera* subspecies and in *C. fraterna*, which is supposed to be one of the wild ancestors of *C. pepo* (Gong *et al.,* 2012). The high susceptibility observed in the whole range of diversity of this species evidences that this virus is a major threat to the cultivation of zucchini.

Despite the high susceptibility of *C. pepo*, the genus *Cucurbita* is highly variable and this variability can be exploited to identify sources of resistance to ToLCNDV in other species. The species *C. maxima*, represented by accessions from its center of origin, Argentina and surrounding countries, and from secondary centers of diversification in Africa (Ferriol *et al*., 2003), showed a delayed infection compared to *C. pepo*. However, disease symptoms evolved from moderate to very severe as infection progressed in most

of the assayed accessions, mainly after whitefly infection. This response is therefore not useful for developing resistant cultivars.

The best results were found in *C. moschata*. This species also displayed the best response in the preliminary screening that we performed to study the host range of the Spanish isolate of ToLCNDV by mechanical transmission (López *et al*., 2015). *Cucurbita moschata* originates from the lowlands of Central America, but within the *Cucurbita* genus is one of the species that became most spread worldwide after European contact (Ferriol *et al*., 2003). Nowadays it is not a major crop, but a staple grown as local landraces in many developing countries of Asia, Africa and the Americas. These local landraces represent a reservoir of genes of interest already used for *C. pepo* breeding (Paris, 2008). In fact, although the assayed collection included accessions from the centre of origin, the accessions with the best responses, remaining nearly symptomless after all inoculations assays, were the Large Cheese improved cultivar from the USA (PI 605406, Burpee Company) and the Indian landrace PI 381814. Similarly, resistance to ToLCNDV in melon was found in Indian accessions (López *et al*., 2015), which can be related with the co-evolution of host and pathogen in this area, in which ToLCNDV was detected for the first time infecting cucurbits many years ago.

The plants of these two accessions of *C. moschata* remained symptomless and with a very low virus titer after whitefly inoculation. Since the whitefly inoculation was not performed using clip cages in individual plants, vector non–preference or antibiosis mechanisms might account for this resistant behaviour. However, the response of these accessions (no or mild symptoms and low viral load) after three rounds of mechanical inoculation, with both field and clone inocula, support the existence of high levels of resistance to the virus.

Finding virus resistance in *C. moschata* is not unexpected as this species has been often used as a source of virus resistance in the *Cucurbita* genus. For example, Nigerian Local is one of the multi-resistant accessions used for *C. pepo* breeding (Brown *et al*., 2003), with reported resistance to *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Papaya ringspot virus W* (PRSV-W) (*Potyvirus*, family *Potyviridae*) and *Cucumber mosaic virus* (CMV, *Cucumovirus*, family *Bromoviridae*). Some of these resistance genes have been used for breeding *C. moschata* and *C. pepo*. In fact, most of the resistance genes of *C. pepo* have been introduced in this species through interspecific crosses. Also, this species includes several sources of moderate

resistance to the begomovirus SLCV in field tests (McCreigth & Kishaba, 1991), whereas *C. pepo, C. fraterna* and *C. maxima* are highly susceptible.

The wild species *C. ecuadorensis*, *C. lundelliana*, *C. foetidisissima* and *C. ockeechobeensis* are potential sources of resistance to SLCV, although they show different behaviour under greenhouse and field tests (McCreigth & Kisaba, 1991). With ToLCNDV, the most promising species after mechanical inoculation were *C. lundelliana* and *C. ockeechobeensis.* However, both developed severe infections after whitefly inoculation. Differences in the response of these wild species after mechanical and whitefly transmission could be due to difficulties in the mechanical inoculation and to the poor adaptation of these species to growth in a climatic chamber.

The lack of clear resistance within the wild *Cucurbita* species enhances the importance of the new selected *C. moschata* accessions, which are good candidates for breeding programs to avoid damage caused by ToLCNDV as they are partially crossable to *C. pepo* (Whitaker and Robinson, 1986). We are now crossing them to susceptible *C. moschata* and to *C. pepo*, to construct segregant populations for inheritance studies and to introgress the resistance into zucchini. The validation of the use of inoculum derived from the infectious clones for resistance screenings will facilitate its use in further genetic studies with segregant populations.

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Table 1. Response of *Cucurbita pepo* accessions to mechanical and whitefly inoculation with ToLCNDV.

Origin	Species/ subespecies/ morphotype	Accession [c]	Mechanical inoculation[a]		Whitefly transmission [b]						
			Symptoms		Symptomatic plants (%)		Symptoms				Plant vigor
			30dpi		21dpi	35dpi	21dpi		35dpi		28dpi
			Mean	Range			Mean	Range	Mean	Range	Mean
	C. pepo										
	subsp. pepo										
Guatemala	Pumpkin	CATIE-11368	3.6	3-4	20	100	0.75	0-2	4.0	4-4	2.0
Turkey	Pumpkin	PI-169462	3.0	2-4	0	100	0.0	0-0	4.0	4-4	2.5
Turkey	Pumpkin	PI-204698	3.3	2-4	0	100	0.0	0-0	4.0	4-4	1.0
Turkey	Pumpkin	PI-171628	4.0	4-4	100	100	2.2	1-3	4.0	4-4	2.4
Italy	Pumpkin	PU-TON	3.6	3-4	100	100	2.3	1-4	4.0	4-4	2.0
Italy	Pumpkin	PU-TOP	3.4	3-4	60	100	0.60	0-1	4.0	4-4	1.0
Spain (Guadalajara)	Pumpkin	359	4.0	4-4	60	100	1.0	0-3	4.0	4-4	1.0
Spain (Cuenca)	Pumpkin	1012	3.0	1-4	80	100	0.80	0-1	4.0	4-4	2.0
Spain (Cuenca)	Pumpkin	1086	3.7	3-4	80	100	1.4	0-3	4.0	4-4	1.2
Spain (Huelva)	Pumpkin	AN-CU-83	4.0	4-4	40	100	0.80	0-3	4.0	4-4	2.8
Spain (Canary Islands)	Pumpkin	CA-CU-43	4.0	4-4	50	100	1.5	0-4	4.0	4-4	1.8
Spain (Canary Islands)	Pumpkin	CA-CU-46	4.0	4-4	100	100	3.8	3-4	4.0	4-4	2.0
Spain (Canary Islands)	Pumpkin	CA-CU-48	2.3	1-4	75	100	1.8	0-4	4.0	4-4	1.0
Spain (Canary Islands)	Pumpkin	CA-CU-57	3.0	2-4	100	100	2.5	1-4	4.0	4-4	2.5
Spain (Canary Islands)	Pumpkin	CA-CU-59	4.0	4-4	25	100	0.25	0-1	4.0	4-4	2.5
Spain (Canary Islands)	Pumpkin	CA-CU-110	3.4	3-4	50	100	2.0	0-4	4.0	4-4	2.5
Spain (Canary Islands)	Pumpkin	CA-CU-192	4.0	4-4	100	100	4.0	4-4	4.0	4-4	1.0
Spain (Canary Islands)	Pumpkin	CA-CU-21	3.0	1-4	100	100	2.5	1-4	4.0	4-4	1.0
Hungary	Pumpkin	IVIA-506	2.4	1-4	100	100	2.5	1-3	4.0	4-4	2.5
Morocco	V. Marrow	AFR-CU-12	4.0	4-4	100	100	2.3	1-4	4.0	4-4	2.5
Morroco	V. Marrow	AFR-CU-8	4.0	4-4	80	100	2.2	0-3	4.0	4-4	2.5
Morroco	V. Marrow	AFR-CU-15	3.2	3-4	100	100	2.5	1-3	4.0	4-4	2.5
Morroco	V. Marrow	AFR-CU-17	4.0	4-4	75	100	1.5	0-3	4.0	4-4	1.5
Morroco	V. Marrow	AFR-CU-22	4.0	4-4	0	100	0.0	0-0	4.0	4-4	2.4
Spain (Guadalajara)	V. Marrow	942	4.0	4-4	40	100	1.0	0-3	4.0	4-4	1.0
Spain (Guadalajara)	V. Marrow	949	4.0	4-4	80	100	1.4	0-3	4.0	4-4	2.8
Spain (Huesca)	V. Marrow	A-CU-12	4.0	4-4	40	100	0.40	0-1	4.0	4-4	2.4

Spain (Almeria)	V. Marrow	AN-CU-113	3.0	2-4	100	100	3.7	3-4	4.0	4-4	2.0
Spain (Cádiz)	V. Marrow	AN-CU-27	4.0	4-4	100	100	4.0	4-4	4.0	4-4	2.0
Spain (Segovia)	V. Marrow	CL-CU-19	3.0	1-4	50	100	0.50	0-1	4.0	4-4	1.3
Spain (Valladolid)	V. Marrow	CL-CU-21	3.2	1-4	50	100	1.5	0-3	4.0	4-4	2.0
Spain (Cuenca)	V. Marrow	CM-CU-32	3.9	3-4	50	100	1.0	0-3	4.0	4-4	2.0
Spain (Cuenca)	V. Marrow	CM-CU-47	3.6	3-4	33	100	0.30	0-1	4.0	4-4	2.0
Spain (Valencia)	V. Marrow	V-CU-10	4.0	4-4	50	100	2.0	0-4	3.3	1-4	2.3
Spain (Alicante)	V. Marrow	V-CU-32	3.3	3-4	100	100	1.0	1-1	4.0	4-4	3.0
Spain (Canary Islands)	V. Marrow	CA-CU-79	4.0	4-4	50	100	2.0	0-4	4.0	4-4	2.5
Spain (Canary Islands)	V. Marrow	CA-CU-82	4.0	4-4	67	100	0.70	0-1	4.0	4-4	2.3
Spain (Canary Islands)	V. Marrow	CA-CU-83	3.8	3-4	100	100	2.5	1-3	4.0	4-4	2.8
Spain (Canary Islands)	V. Marrow	CA-CU-84	4.0	4-4	50	100	0.50	0-1	4.0	4-4	2.3
Spain (Canary Islands)	V. Marrow	CA-CU-113	3.4	2-4	100	100	4.0	4-4	4.0	4-4	1.5
Spain (Valencia)	V. Marrow	IVIA-032	4.0	4-4	50	100	0.50	0-1	4.0	4-4	0.80
Spain (Teruel)	V. Marrow	A-CU-2	3.3	2-4	75	100	1.5	0-3	4.0	4-4	2.3
Spain (Tarragona)	V. Marrow	C-CU-3	3.5	3-4	75	100	2.5	0-4	4.0	4-4	2.5
Ecuador	Zucchini	ECU-227	3.7	2-4	50	100	1.3	0-4	4.0	4-4	2.0
Italy	Zucchini	ZU-NVM	2.7	2-4	60	100	0.60	0-1	4.0	4-4	1.8
Spain (Guadalajara)	Zucchini	435	4.0	4-4	60	100	0.80	0-1	4.0	4-4	1.0
Spain (Huesca)	Zucchini	A-CU-13	2.6	2-4	0	100	0.0	0-0	4.0	4-4	1.8
Spain (Caceres)	Zucchini	E-CU-10	4.0	4-4	67	100	0.70	0-3	4.0	4-4	2.0
Spain (Caceres)	Zucchini	E-CU-27	4.0	4-4	100	100	1.7	1-3	4.0	4-4	2.8
Spain (Murcia)	Zucchini	MU-CU-20	3.1	3-4	75	100	1.75	0-4	4.0	4-4	3.0
Spain (Murcia)	Zucchini	MU-CU-16	4.0	4-4	50	100	0.50	0-1	4.0	4-4	2.3
Spain (Córdoba)	Cocozelle	AN-CU-75	3.7	2-4	60	100	1.8	0-4	4.0	4-4	1.0
Spain (Barcelona)	Cocozelle	C-CU-9	4.0	4-4	60	100	1.4	0-3	4.0	4-4	1.6
Spain (Castellón)	Cocozelle	PAS-15834	3.4	2-3	75	100	2.5	0-4	4.0	4-4	1.5
Spain (Castellón)	Cocozelle	PASCUAL-40	3.0	3-3	50	100	1.5	0-4	4.0	4-4	2.8
Spain (Alicante)	Cocozelle	V-CU-185	3.6	3-4	100	100	4.0	4-4	4.0	4-4	2.0
Spain (Valencia)	Cocozelle	V-CU-74	3.6	3-4	100	100	2.3	1-3	4.0	4-4	2.3
Italy	Cocozelle	CO-DBT	4.0	4-4	100	100	2.8	1-4	4.0	4-4	2.0
Italy	Cocozelle	CO-LBS	2.6	1-4	67	100	2.3	0-4	4.0	4-4	2.0
Italy	Cocozelle	CO-LUF	2.5	1-4	100	100	2.3	1-3	4.0	4-4	2.0
Italy	Cocozelle	CO-ROM	4.0	4-4	40	100	1.0	0-3	4.0	4-4	2.0
Italy	Cocozelle	CO-SPQ	4.0	4-4	100	100	1.7	1-3	4.0	4-4	1.0
Italy	Cocozelle	CO-VAL	4.0	4-4	100	100	3.5	1-4	4.0	4-4	3.0
Greece	Cocozelle	Grecia-6	3.6	2-4	40	100	0.90	0-4	4.0	4-4	2.8
	subsp. ovifera										
USA	Croockneck	NSL-5206	3.8	2-4	100	100	3.3	3-4	4.0	4-4	1.0
USA	Croockneck	NSL-5227	4.0	4-4	50	100	1.3	0-4	4.0	4-4	1.8
USA	Croockneck	PI-106681	3.3	3-4	25	100	0.25	0-1	4.0	4-4	1.5
USA	Croockneck	USA-CU-2	4.0	4-4	100	100	2.8	1-4	4.0	4-4	1.8
USA	Acorn	PI-615111	4.0	4-4	60	100	1.6	0-3	4.0	4-4	1.8
USA	Acorn	PI-518687	3.4	2-4	25	100	0.30	0-1	4.0	4-4	1.3

Spain (Valencia)	Scallop	V-CU-196	4.0	4-4	100	100	1.0	1-1	4.0	4-4	2.0
Spain (Valencia)	Ornamental	V-CU-81	3.7	3-4	20	100	0.40	0-1	4.0	4-4	1.4
Spain (Valencia)	Ornamental	IVIA-569	4.0	4-4	25	100	1.0	0-4	4.0	4-4	2.8
F1	hybrid	TFxPI-171628	4.0	4-4	50	100	1.5	0-4	4.0	4-4	2.0
F1	hybrid	TFx V-CU-196	4.0	4-4	50	100	2.0	0-4	4.0	4-4	2.3
	C. fraterna										
Mexico		PI-614701	3.6	3-4	0	100	0.0	0-0	3.0	3-3	0.0
Mexico		PI-532354	4.0	4-4	100	100	1.0	1-1	4.0	4-4	1.0

[a] Mean and range of symptoms scored in ten plants per genotype mechanically inoculated with a ToLCNDV isolate originally identified in infected zucchini plants from Almeria, according to the following scale: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms or plant death (López et al., 2015).

[b] Percentage of plants showing symptoms of ToLCNDV after whitefly transmission in the greenhouse experiment. Mean and range of symptoms were scored in twelve plants per genotype (two sets of six plants). Mean vigor of the plants of each genotype was scored from 0 (weak plants) to 4 (highly vigorous).

[c] Most of the assayed accessions are from the germplasm collection of the Institute for Conservation and Breeding of the Agrodiversity, Spain (COMAV-UPV). PI and NSL genotypes were kindly provided by U.S. Dept. Agric. National Plant Germplasm System and CATIE by the Centro Agronómico Tropical de Investigación y Enseñanza, Costa Rica.

Table 2. Response of *Cucurbita* spp. accessions to mechanical and whitefly inoculation with ToLCNDV.

Origin	Species/ subespecies/ morphotype	Accession [c]	Mechanical inoculation [a]		Whitefly transmission [b]						
			Symptoms		Symptomatic plants (%)		Symptoms				Plant vigour
			30dpi		21dpi	35dpi	21dpi		35dpi		28dpi
			Mean	Range			Mean	Range	Mean	Range	Mean
	C. maxima										
Argentina		SUD-CU-6	3.6	2-4	25	100	1.0	0-4	4.0	4-4	3.0
Argentina		MAX-306	2.6	1-4	0	100	0.0	0-0	4.0	4-4	2.0
Argentina		BGV-15415	4.0	4-4	0	100	0.0	0-0	4.0	4-4	2.8
Bolivia		PI-543227	2.2	1-4	50	100	0.69	0-1	4.0	4-4	2.0
Chile		VAV-3202	3.4	1-4	100	100	2.0	1-3	4.0	4-4	2.1
Colombia		CATIE-9824	2.6	0-4	50	100	1.0	0-3	4.0	4-4	2.8
Peru		VAV-4273	2.8	1-4	0	100	0.0	0-0	4.0	4-4	2.2
Morocco		AFR-CU-1	3.0	2-4	0	100	0.0	0-0	4.0	4-4	3.2
Morocco		AFR-CU-8	3.4	3-4	0	100	0.0	0-0	4.0	4-4	2.0
Morocco		AFR-CU-18	3.6	2-4	0	100	0.0	0-0	4.0	4-4	2.0
Angola		AFR-CU-38	3.8	2-4	0	100	0.0	0-0	4.0	4-4	2.1
Angola		AFR-CU-73	2.3	1-4	20	100	0.0	0-1	4.0	4-4	2.1
African Republic		VAV-2422	3.1	2-4	0	100	0.0	0-0	4.0	4-4	2.0
Spain (Jaen)		AN-CU-59	3.0	1-4	20	100	1.0	0-4	4.0	4-4	2.8
	C. moschata										
Costa Rica		PI-369346	2.5	1-4	0	100	0.0	0-0	4.0	4-4	1.0
Cuba		SUD-CU-8	3.6	3-4	20	80	0.30	0-1	3.2	0-4	1.0
Ecuador		ECU-46	1.7	0-4	0	100	0.0	0-0	4.0	4-4	2.3
Guatemala		PI-264551	2.6	2-4	20	75	0.30	0-1	3.0	0-4	1.0
Dom. Republic		SUD-CU-	2.8	2-4	0	100	0.0	0-0	3.0	1-4	1.0

USA		13 PI-604506	0.70	0-1	0	0	0.0	0-0	0.0	0-0	2.0
Canada		PI-550689	1.2	1-4	0	50	0.0	0-0	1.5	0-3	1.2
Zimbawe		PI-482527	4.0	4-4	0	100	0.0	0-0	2.7	0-4	1.3
Nigeria		Nig.Local	0.60	0-1	60	40	0.80	0-1	1.7	0-4	2.0
Spain (Canary Islands)		CA-CU-26	1.3	0-2	0	100	0.0	0-0	4.0	4-4	3.0
Spain (Jaen)		AN-CU-45	1.4	0-3	0	60	0.0	0-0	2.4	0-4	1.6
Spain (Valencia)		IVIA-205	1.8	1-3	0	100	0.0	0-0	4.0	4-4	2.0
Japan		KUROKAWA	0.40	0-1	0	75	0.0	0-0	1.8	0-4	1.0
India		PI-381814	0.20	0-1	0	0	0.0	0-0	0.0	0-0	2.0
	Other species										
Peru	***C. ficifolia***	GRIF9448	4.0	4-4	100	100	3.0	3-3	4.0	4-4	1.0
Belize	***C. lundeliana***	PI-438542	0.60	0-2	0	100	0.0	0-0	4.0	4-4	0.5
Mexico	***C. foetidisima***	PI-442197	3.0	2-4	0	100	0.0	0-0	3.8	3-4	1.0
Mexico	***C. okeechobeensis***	PI-532363	0.0	0-0	100	100	1.0	1-1	4.0	4-4	1.0
Mexico	***C. okeechobeensis***	PI-512105	2.0	1-4	60	100	0.80	0-1	4.0	4-4	0.8

[a] Mean and range of symptoms scored in ten plants per genotype mechanically inoculated with a ToLCNDV isolate originally identified in infected zucchini plants from Almeria, according to the following scale: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms or plant death (López et al., 2015).

[b] Percentage of plants showing symptoms of ToLCNDV after whitefly transmission in the greenhouse experiment. Mean and range of symptoms were scored in twelve plants per genotype (two sets of six plants). Mean vigor of the plants of each genotype was scored from 0 (weak plants) to 4 (highly vigorous).

[c] Most of the assayed accessions are from the germplasm collection of the Institute for Conservation and Breeding of the Agrodiversity, Spain (COMAV-UPV). PI and NSL genotypes were kindly provided by U.S. Dept. Agric. National Plant Germplasm System and CATIE by the Centro Agronómico Tropical de Investigación y Enseñanza, Costa Rica.

Table 3. Confirmation of the response of selected *Cucurbita* genotypes to mechanical inoculation with two inoculum sources of ToLCNDV.

Species/Accession	Inoculum from field infected plants				Inoculum from agroinoculated plants			
	15dpi		30dpi		15dpi		30dpi	
	Mean [a]	% PCR positive plants [b]	Mean (range)	% PCR positive plants	Mean	% PCR positive plants	Mean (range)	% PCR positive plants
C. maxima								
CATIE-9824	1.8	60	2.4 (0-4)	100	1.0	60	1.6 (0-4)	80
PI-543227	1.6	100	2.2 (1-4)	100	1.8	80	3.2 (1-4)	80
AFR-CU-1	2.0	100	2.6 (1-4)	100	1.8	100	3.0 (2-4)	80
VAV-4273	1.3	80	2.7 (1-4)	100	3.2	80	3.2 (2-4)	100
AFR-CU-18	2.4	100	3.0 (2-4)	100	2.7	100	4.0 (4-4)	100
AFR-CU-38	3.6	100	3.8 (3-4)	100	2.4	80	3.4 (2-4)	80
C. moschata								
PI-381814	0.0	100	0.20 (0-1)	100	0.0	80	0.0 (0-0)	80
PI-604506	0.80	100	0.60 (0-1)	100	0.0	80	0.0 (0-0)	80
NIGERIAN LOCAL	0.60	60	0.60 (0-1)	80	0.0	80	0.0 (0-0)	80
KUROKAWA	0.25	80	0.20 (0-1)	100	0.0	40	0.0 (0-0)	60
AN-CU-45	0.60	100	0.80 (0-1)	100	0.20	60	1.4 (0-3)	100
IVIA-205	1.5	80	1.5 (0-4)	100	2.0	60	2.0 (0-4)	80
PI-550689	0.80	100	1.2 (0-4)	100	0.50	20	2.4 (0-4)	60
PI-369346	2.3	100	2.6 (1-4)	100	1.6	80	1.7 (0-4)	60
ECU-46	0.30	60	1.7 (1-4)	100	1.0	100	2.7(3-4)	100
PI-482527	3.0	100	4.0 (4-4)	100	4.0	100	4.0 (4-4)	100
SUD-CU-8	2.7	100	3.2 (2-4)	100	1.6	100	3.0 (2-4)	100
C. pepo								

IVIA-506	2.7	100	2.7 (0-4)	100	2.0	100	3.0 (0-4)	100
CM-CU-37	2.6	100	3.6 (2-4)	100	2.5	100	3.5 (2-4)	100
CATIE-11368	3.6	100	3.8 (3-4)	100	3.0	100	4.0 (4-4)	100
359	4.0	100	4.0 (4-4)	100	4.0	100	4.0 (4-4)	100
C. fraterna								
PI-614701	3.8	100	4.0 (4-4)	100	1.8	100	4.0 (4-4)	100
C. ficifolia								
GRIFF-9448	4.0	100	4.0 (4-4)	100	4.0	100	4.0 (4-4)	100
C. lundeliana								
PI-438542	0.20	75	0.40 (0-2)	100	0.80	40	0.20 (0-1)	60
C. foetidissima								
PI-442197	2.0	100	3.0 (2-4)	100	2.8	60	2.0 (1-4)	100
C. okeechobeensis								
PI-512105	1.2	100	1.7 (1-4)	100	1.0	80	1.5 (1-4)	100

[a] Mean and range of symptoms scored in five plants per genotype mechanically inoculated with field inoculum (ToLCNDV isolate originally identified in infected zucchini plants from Almeria) and with an infectious clone (a ToLCNDV isolate from Almeria, cloned into *Agrobacterium* and transmitted to susceptible zucchini plants), according to the following scale: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms or plant death.
[b] Percentage of plants that were PCR positive using standard PCR.

Figure captions

Figure 1. Symptoms of ToLCNDV in *Cucurbita* species. **A.** Typical symptoms of curling and severe mosaic of young leaves and short internodes observed in *C. pepo* (scored as 4, severe symptoms). **B.** Characteristic symptoms of leaf decay and intense yellowing found in most accessions of *C. maxima*. **C.** Symptoms of leaf mottling evolving to severe mosaic in the susceptible accessions of *C. moschata*.

Figure 2. Time course response of accessions belonging to the three main cultivated *Cucurbita* species (*C. pepo, C. moschata* and *C. maxima*) to whitefly inoculation with ToLCNDV, measured as percentage of symptomatic plants **(A)** and mean symptom score **(B)** (on a scale from 0, symptomless, to 4, very severe symptoms). Means of 12 plants (two sets of six plants) are shown. Detailed information of the different *C. pepo* subsp. *pepo* morphotypes is included (P: Pumpkin; VM: Vegetable Marrow; Z: Zucchini; C: Cocozelle) and is compared to the subsp. *ovifera* **(C)** and the wild relative *C. fraterna* **(D).**

Figure 3. Relative quantification of ToLCNDV in the apex of infected plants at 28 dpi by qPCR. The measurements were performed in several genotypes of *C. pepo*, *C. maxima* and *C. moschata* with different responses to ToLCNDV after whitefly inoculation. Two pools of six plants each were independently amplified and three technical replications were done on each pool (mean and standard errors are included in the figure). The mean symptom scores at 28 and 35 dpi after whitefly inoculation are shown in parentheses after the accession´s name.

Figure 4. Relative quantification of ToLCNDV in the apex of infected plants at 15 and 30 dpi (mechanical inoculation using field inoculum) by qPCR. The measurements were performed in the two resistant accessions of *C. moschata*, in one accession of the same species with severe symptoms at the end of the assay, and in one *C. pepo* susceptible control. Three plants per accession were analyzed and three technical replications were done on each sample (mean and standard errors are included in the figure). The mean symptom scores at 15 and 30 dpi after mechanical inoculation are shown.

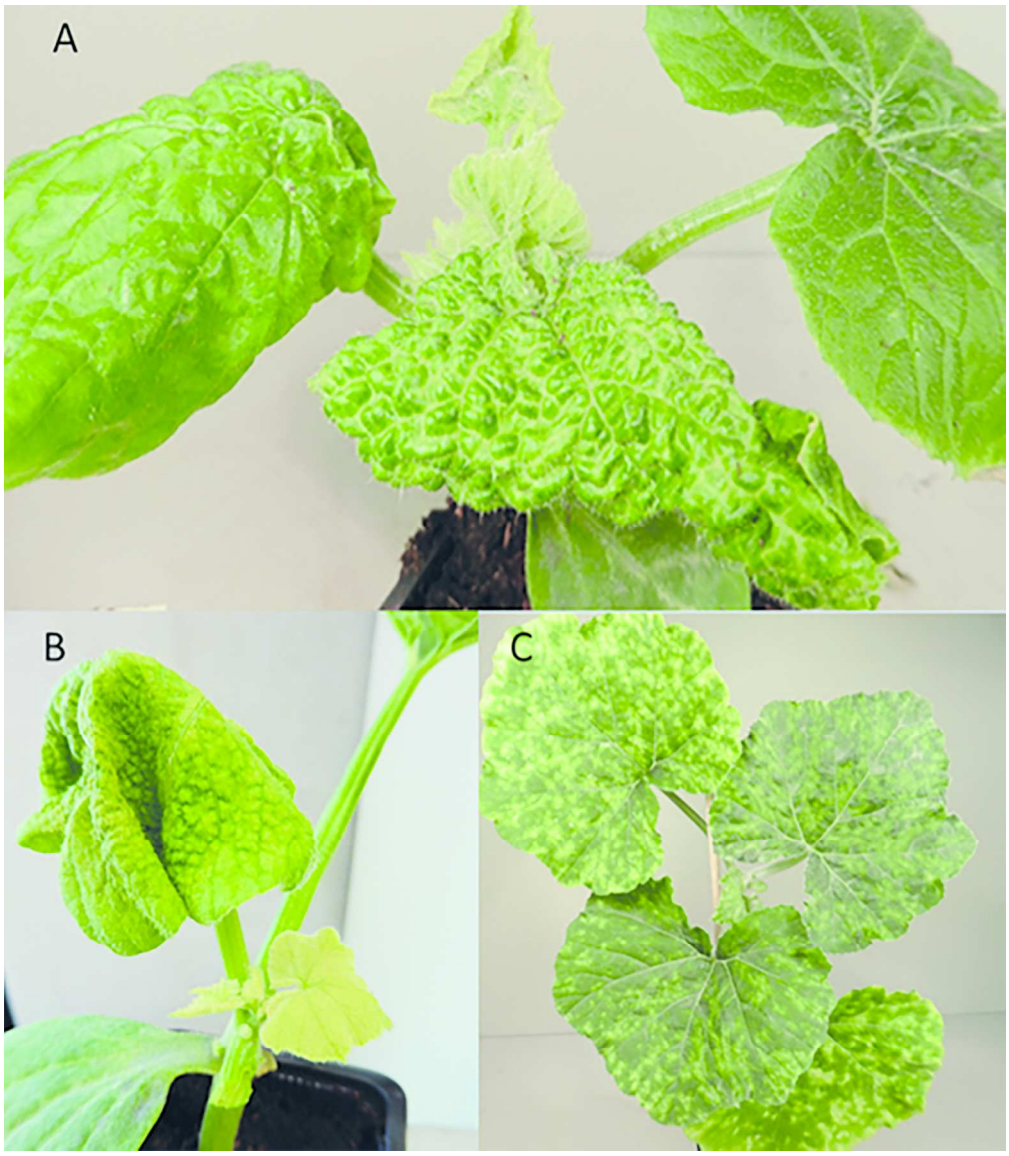

Figure 1. Symptoms of ToLCNDV in Cucurbita species. A. Typical symptoms of curling and severe mosaic of young leaves and short internodes observed in C. pepo (scored as 4, severe symptoms). B. Characteristic symptoms of leaf decay and intense yellowing found in most accessions of C. maxima. C. Symptoms of leaf mottling evolving to severe mosaic in the susceptible accessions of C. moschata.
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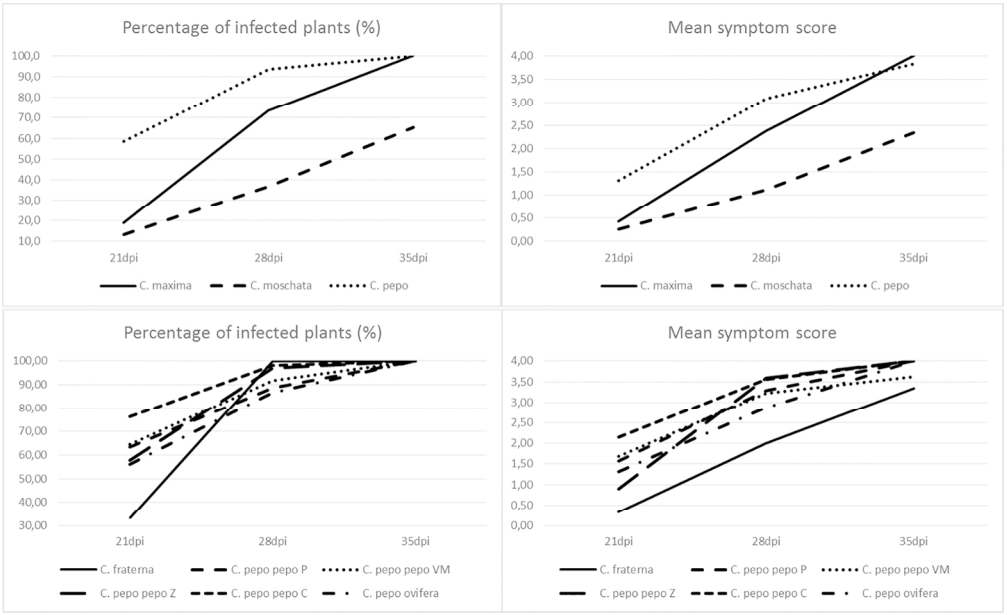

Figure 2. Time course response of accessions belonging to the three main cultivated Cucurbita species (C. pepo, C. moschata and C. maxima) to whitefly inoculation with ToLCNDV, measured as percentage of symptomatic plants (A) and mean symptom score (B) (on a scale from 0, symptomless, to 4, very severe symptoms). Means of 12 plants (two sets of six plants) are shown. Detailed information of the different C. pepo subsp. pepo morphotypes is included (P: Pumpkin; VM: Vegetable Marrow; Z: Zucchini; C: Cocozelle) and is compared to the subsp. ovifera (C) and the wild relative C. fraterna (D).
170x103mm (300 x 300 DPI)

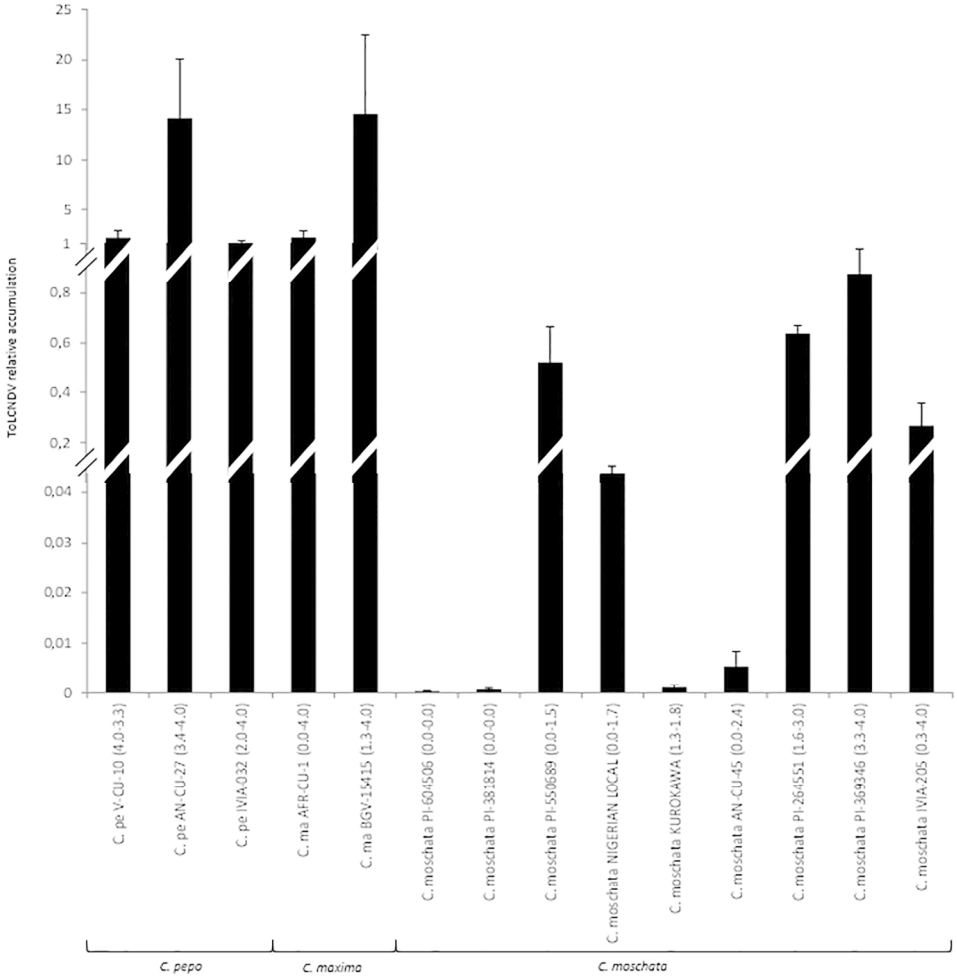

Figure 3. Relative quantification of ToLCNDV in the apex of infected plants at 28 dpi by qPCR. The measurements were performed in several genotypes of C. pepo, C. maxima and C. moschata with different responses to ToLCNDV after whitefly inoculation. Two pools of six plants each were independently amplified and three technical replications were done on each pool (mean and standard errors are included in the figure). The mean symptom scores at 28 and 35 dpi after whitefly inoculation are shown in parentheses after the accession´s name.
170x170mm (300 x 300 DPI)

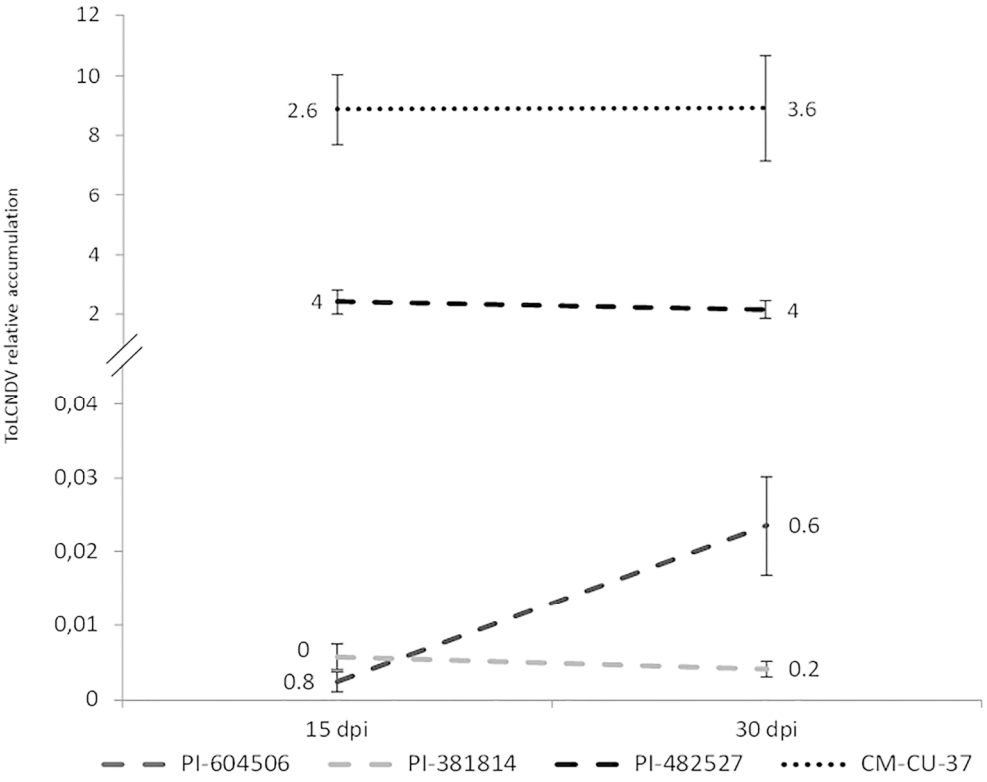

Figure 4. Relative quantification of ToLCNDV in the apex of infected plants at 15 and 30 dpi (mechanical inoculation using field inoculum) by qPCR. The measurements were performed in the two resistant accessions of C. moschata, in one accession of the same species with severe symptoms at the end of the assay, and in one C. pepo susceptible control. Three plants per accession were analyzed and three technical replications were done on each sample (mean and standard errors are included in the figure). The mean symptom scores at 15 and 30 dpi after mechanical inoculation are shown.
170x140mm (300 x 300 DPI)