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Short running title: Inoculation and detection of ToLCNDV infection in cucurbits

Inoculation of cucumber, melon and zucchini varieties with *Tomato leaf curl New Delhi virus* (ToLCNDV) and evaluation of infection using different detection methods

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Abstract

The disease caused by *Tomato leaf curl New Delhi virus* (ToLCNDV), which is naturally transmitted by the whitefly *Bemisia tabaci*, causes important economic losses in cucurbit crops. The availability of simple and efficient inoculation protocols and detection methods is necessary for screening varieties and germplasm collections as well as for breeding populations. We evaluated the infectivity of ToLCNDV inocula prepared using three different buffers for mechanical sap inoculation in a susceptible variety of zucchini. We found that inoculum prepared with buffer III, which contains polyvinylpyrrolidone, is highly efficient for mechanical inoculation, with 100% of plants displaying severe symptoms 21 days post-inoculation. Using this buffer, we mechanically inoculated 19 commercial varieties of cucurbit crops (six of cucumber, six of melon and seven of zucchini), evaluated the evolution of symptoms and diagnosed infection using nine different ToLCNDV detection methods (four based on serology, four on molecular hybridization, and one based on PCR detection). The results revealed that all varieties are susceptible, although cucumber varieties display less severe symptoms than those of melon or zucchini. All detection methods were highly efficient (more than 85% of plants testing positive) in melon and zucchini, but in cucumber, the percentage of positive plants detected with serology and molecular hybridization methods ranged from 20.4% with *Squash leaf curl virus* (SLCV) antiserum, to 78.5% with DNA extract hybridization. Overall, the best detection results were obtained with PCR, with 92.6%, 92.4% and 98.4% cucumber, melon and zucchini plants, respectively, testing positive. When considering the overall results in the three crops, the best serology and molecular hybridization methods were those using *Watermelon chlorotic stunt virus* (WmCSV) antiserum and DNA extract, respectively. The inoculation methodology developed and the information

on detection methods are of great relevance for the selection and breeding of varieties of cucurbit crops that are tolerant or resistant to ToLCNDV.

Keywords

Cucurbits, molecular hybridization, PCR, sap inoculation, serology, ToLCNDV

Introduction

Viruses may dramatically affect crop productivity and are a major limiting factor in the profitability of many horticultural crops. Recently, the outbreak of emerging viruses, such as ToLCNDV in cucurbits (Juárez et al., 2014), has caused great concern among producers. In cucumber (*Cucumis sativus* L.), ToLCNDV infection causes interveinal yellowing, vein banding and wrinkled veins in the leaves, although the fruits may remain symptomless (Font and Alfaro-Fernández, 2015). ToLCNDV-associated symptoms in melon (*Cucumis melo* L.) and zucchini (*Cucurbita pepo* L. subsp. *pepo*) consist of curling, severe mosaic of young leaves and vein swelling, short internodes and fruit skin roughness (Juárez et al., 2014). In the last two species, symptoms in infected plants depend on the timing of infection. In late infections, the young leaves curl and pucker, usually towards the adaxial surface but sometimes towards the abaxial surface, and they commonly show yellowing, which is more intense in the youngest leaves. Infected plants stop growing, and young fruits usually display skin roughness. This symptom is accentuated in mature fruits. When infection occurs in early stages, severe curling and puckering are observed in young leaves, the growth is dramatically impaired, and total abortion of fruits occurs.

ToLCNDV belongs to the genus *Begomovirus* (family *Geminiviridae*). This virus has two circular single-stranded DNA genomes of approximately 2.7 kb each (DNA-A

and DNA-B), which are encapsidated in geminate particles (Papidam et al., 1995; Fauquet et al., 2008; Ito et al., 2008). ToLCNDV is transmitted in a persistent and circulative manner by the silverleaf whitefly (*Bemisia tabaci* Genn.) (Chang et al., 2010). Although some viruses in the same family can be transmitted to the next whitefly generation (Ghanim et al., 1998), it remains to be determined whether this is also true for ToLCNDV. Additionally, it has not yet been determined whether it can be transmitted by rubbing or by seed.

Different serological and molecular detection methods may be used to diagnose ToLCNDV. As the capsid protein of begomoviruses is highly conserved (Fondong, 2013), antisera generated against other closely related species of this genus such as *Tomato yellow leaf curl virus* (TYLCV), *Squash leaf curl virus* (SLCV) or *Watermelon chlorotic stunt virus* (WmCSV) may recognize ToLCNDV. PCR assays with specific primers for ToLCNDV or molecular hybridization with specific probes have also been described as adequate detection methods (Gawande et al. 2007; López et al., 2015; Ruiz et al. 2015; Alfaro-Fernández et al. 2016)

Unfortunately, the control measures for the disease caused by ToLCNDV are very limited. Elimination of affected plants, avoidance of overlapping susceptible crops, and the control of whitefly populations are cultural practices recommended to decrease the disease spread (Lecoq and Katis, 2014). Genetic resistance would be the best strategy for control (Sáez et al., 2016). In this sense, the availability of an efficient inoculation method is necessary in breeding programmes that screen sources of resistance and segregating populations. In this respect, ToLCNDV has been mechanically inoculated in several cucurbit crops (Chang et al., 2010, Sohrab et al., 2013). Recently, mechanical sap transmission with an inoculation buffer containing 2-mercaptoethanol and polyvinylpyrrolidone (PVP) from ToLCNDV infected zucchini plants to cucumber,

melon, watermelon and pumpkins has been reported (López et al., 2015). However, in other cases, mechanical inoculation failed (Samretwanich et al., 2000; Chang et al., 2010).

The availability of an efficient method for mechanical inoculation and a useful, rapid, economic and reliable diagnostic method is very important for the identification of sources of ToLCNDV resistance in cucurbit crops. In the present work, an inoculation procedure for mechanical sap transmission of ToLCNDV was developed and several detection techniques for this begomovirus are evaluated in a collection of commercial cucumber, melon and zucchini varieties inoculated with ToLCNDV.

Materials and methods

Experiment 1. Determination of an efficient mechanical inoculation procedure

Virus source

A ToLCNDV isolate collected from naturally virus-infected zucchini plants in Paraje Lo Soler, Murcia (southern Spain) displaying curling, vein puckering and severe dark-green and light yellow-green mosaic in young leaves was used as the original source of inoculum. The initial inoculum was tested for the presence of potentially existing viruses in the area (Juárez et al., 2013) by means of ELISA against *Cucumber mosaic virus* (CMV) (Soler et al., 2010) and by PCR using specific primers for ToLCNDV (López et al., 2015) and *Cucumber vein yellowing virus* (CVYV) (Picó et al., 2005). All tests except for ToLCNDV were negative, which gave the expected PCR amplification product with a strong signal. The ToLCNDV isolate was transmitted to zucchini seedlings using virus-

free reared silverleaf whiteflies, to avoid potential contamination of other non-tested co-infecting mechanically transmitted viruses. To confirm the absence of other whitefly transmitted viruses, the infected zucchini plants were evaluated again using PCR against CVYV, which is the only other whitefly transmitted virus occasionally affecting *Cucurbita* plants in the area where our isolate was collected (Juárez et al., 2013). The results were negative. Moreover, no symptoms other than those specific to a ToLCNDV infection in zucchini (Sáez et al., 2016) were observed. The ToLCNDV isolate was designated ND2014-1V.

Susceptible zucchini (variety Z6) plants were used for determining the best buffer for inoculum preparation. This zucchini variety is highly susceptible to the virus under field conditions. Zucchini seeds were soaked in 5% sodium hypochlorite for 3 minutes, subsequently rinsed in distilled water, and sown in Petri dishes containing a layer of hydrophilic cotton covered by a layer of filter paper and kept at 25 ºC for germination.

Inoculum preparation

Inoculum was prepared using a 1:4 (w:v) proportion of infected leaves:buffer. The leaves, displaying severe symptoms of ToLCNDV infection, were obtained from zucchini plantlets inoculated with the ND2014-1V ToLCNDV isolate using virus-free reared silverleaf whiteflies and maintained in an insect-free climatic chamber (Juárez et al., 2014). The leaves were cut into small pieces and mixed; from this bulk, samples were taken for the inoculum preparation. Three different buffers were tested in this experiment: I) 0.1 M sodium phosphate (pH=7.2), 0.04% β-mercaptoethanol, 0.2% Na_2SO_3 and 2% Celite; II) 0.1 M sodium phosphate (pH=7.2), 0.2% sodium sulphite and 2% Celite; and III) 15 mM NaCl, 7.5 mM Na_2HPO_4, 0.38 mM NaH_2PO_4 and 25 mM polyvinylpyrrolidone (PVP-40) (pH=7.2-7.4). Buffers I and II have been used for

mechanical inoculation of ToLCNDV (Sohrab et al., 2013), while buffer III is routinely used in our lab for mechanical inoculation of *Pepino mosaic virus* (PepMV), *Tomato mosaic virus* (ToMV), and *Tomato spotted wilt virus* (TSWV).

Mechanical inoculation and symptoms evaluation

When cotyledons of Z6 germinated zucchini seeds were completely developed, plantlets were transplanted to pots (8 x 8 x 9 cm) filled with commercial growing substrate and placed in a growth chamber under a photoperiod and temperature regime of 16 h light (25 ºC):8 h dark (18 ºC). Plants were subjected to a light irradiance of 65 to 85 $\mu mol \cdot s^{-1} \cdot m^{-2}$ from Sylvania Gro-Lux (Havells Sylvania, Erlangen, Germany) fluorescent tubes. For sap inoculation of ToLCNDV, 1 g of zucchini leaves from infected plants was ground with a mortar and a pestle in 4 mL of inoculation buffer I, II or III. The resulting homogenate was used for inoculation. Plants were inoculated at the two true developed leaves stage by gently rubbing the adaxial surface of the leaf with a cotton swab soaked with the crude homogenate. A second inoculation was performed 7 days after the first inoculation. Control plants were mock inoculated on both dates. For each buffer fifteen plants were used for inoculation, as well as for the control treatment, making a total of 60 plants.

Plants were evaluated for ToLCNDV symptoms at 7, 14, 21 and 28 days post second inoculation (DPI). Symptoms were assessed by visual evaluation using the following scale (López et al., 2015): 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms (Figure 1).

Data analysis

For each combination of DPI date and inoculation buffer, we calculated the percentage of plants displaying symptoms as $100 \times [(n_1 + n_2 + n_3 + n_4)/(n_0 + n_1 + n_2 + n_3 + n_4)]$, and the mean symptom score as $(n_1 + 2 \cdot n_2 + 3 \cdot n_3 + 4 \cdot n_4)/(n_0 + n_1 + n_2 + n_3 + n_4)$, where n_0, n_1, n_2, n_3, and n_4 are the number of plants with symptom score values of 0, 1, 2, 3, and 4, respectively. This mean score, which provides a single averaged value of the symptoms for a treatment and which has a maximum value of 4, is directly proportional to the vulnerability index (VI) used by other authors (Islam et al., 2010, 2011, López et al., 2015), which has a maximum value of 100%. In our case, where five symptom scores are used, VI=25×mean symptom score. The calculation of the percentage of plants displaying symptoms and mean score for each buffer treatment was based on 15 individual plants.

Experiment 2. Comparison of diagnostic techniques and screening of cucumber, melon and zucchini varieties

Virus source and inoculum preparation

The source of inoculum for Experiment 2 was ToLCNDV ND2014-1V, the same used for Experiment 1. Mechanical inoculation was performed using buffer III, also as indicated in Experiment 1.

Plant material and inoculation

Plant materials consisted of six commercial varieties of cucumber (C1 to C6; corresponding to Slicing [C1, C2, C5 and C6] and Pickling [C3 and C4] varietal types), six varieties of melon (M1 to M6; corresponding to Rochet [M1], Tendral [M2], Galia [M3], Pinyonet [M4], Yellow Canary [M5] and Piel de sapo [M6] varietal types) and

seven varieties of zucchini (Z1 to Z7; corresponding to Elongated [Z1 to Z6] and Round [Z7] varietal types). They correspond to traditional varieties grown in Spain. Seed germination, plant cultivation and inoculation were performed as indicated in Experiment 1. Due to the lack of uniformity in seed germination, the number of plants tested per variety ranged between four and nine, with each plant representing one experimental unit.

Diagnostic techniques

Plants were evaluated for symptoms at 7, 14, 21 and 28 DPI as described in Experiment 1. In addition, we assessed three different diagnostic techniques (serological detection, molecular hybridization, and PCR detection) to detect the presence of ToLCNDV in the studied collection. For serological detection four antisera were compared: a polyclonal antiserum for ToLCNDV (DAS-ELISA) (DSMZ, Leibniz-Institut DSMZ GmbH, Braunschweig, Germany), and monoclonal antisera for TYLCV (TAS-ELISA) (Loewe Biochemica GMBH, Sauerlach, Germany), SLCV (TAS-ELISA) (DSMZ) and WmCSV (TAS-ELISA) (DSMZ). Absorbance after serological reaction was measured at 405 nm with a microplate reader (model 550, Biorad, Hercules, California). A sample was considered positive (infected) when the absorbance was higher than the mean absorbance of the mock-inoculated controls plus three times their standard deviation.

For molecular hybridization, a digoxigenin labelled riboprobe specific for a ToLCNDV replication associated gene was evaluated (Alfaro-Fernández et al., 2016). A total volume of 0.7 µL from four different extracts was applied onto a nylon hybridization membrane. The extracts were prepared by grinding plant tissue in a proportion 1:20 (w/v) with three different extraction buffers: A) sample buffer Loewe III for TAS-ELISA of TYLCV (Loewe Biochemica), B) sample extraction buffer for DAS-ELISA of WmCSV, SCLV and ToLCNDV (DSMZ), C) CTAB extraction buffer described by Doyle and

Doyle (1990), in addition to D) undiluted DNA extract consisting of 0.7 µL of the DNA extraction as described below. Each plant of the assay was analysed at 14 and 28 DPI. Non-radioactive nucleic acids spot hybridization was performed as described in Alfaro-Fernández et al. (2016). For PCR-based detection of ToLCNDV, DNA was extracted from apical leaves of each plant at 14 and 28 DPI using the CTAB method (Doyle and Doyle, 1990). DNA was quantified using a Nanodrop 1000 spectrophotometer and later diluted with milli-Q water to a final concentration of 50 ng/µL. PCR assays were carried out in a 15 µL of prepared mixture with appropriate buffer containing 50 ng of total DNA, 0.5 mM MgCl, 5 µM of each primer To-1F and To-1R (López et al., 2015), 5 mM dNTPs and 1 U Taq DNA polymerase.

The PCR conditions were set as follows: 5 min at 94 ºC as an initial denaturation step followed by 35 cycles of 30 s at 94 ºC, 45 s at 55 ºC and 45 s at 72 ºC, with a final extension of 10 min at 72 ºC. The PCR product was approximately 500 bp and was analysed by electrophoresis in 1 % agarose gels in TAE buffer (Tris acetate-EDTA buffer), and stained with ethidium bromide and visualized under UV light.

Data analysis

For each variety and DPI date the percentage of symptomatic plants and the mean symptom score values were calculated as indicated in Experiment 1. For the different diagnostic techniques, the percentage of plants testing positive at 14 and 28 DPI was calculated for each variety. Mean values for species and their standard error were calculated from average variety values.

Results and discussion

Mechanical inoculation protocol

Considering that the source of inoculum and, consequently, the viral concentration used for preparing the inoculation buffers were the same, the results observed for the highly susceptible zucchini variety Z6 suggest that buffer III should be recommended for mechanical inoculation of ToLCNDV (Table 1). Plants inoculated with inoculum prepared with buffer III developed more severe symptoms than those inoculated with buffer I (Sohrab et al., 2013). At 7 DPI, the percentage of symptomatic Z6 plants inoculated with inoculum prepared with buffer III was 73.3%, while for buffer I, it was 40.0%. At 21 DPI, 100% of plants were symptomatic with buffer III, while with buffer I, it required 7 more days for all plants to develop symptoms. The levels of symptomatic plants observed with the inoculum prepared with buffer III were higher than those reported by López et al. (2015) on susceptible zucchini accession MU-CU-16. Additionally, at all dates the mean symptom score for buffer III was higher than that of buffer I. The mean symptom score at 7 DPI was more than twice in plants inoculated with buffer III (1.53) than in those inoculated with buffer I (0.73) (Table 1). In this respect, López et al. (2015) also used a buffer (COMAV buffer) that gave better results than those with buffer I (Sohrab et al., 2013). Unfortunately, we did not test the COMAV buffer, as its composition had not yet been published at the time our Experiment 1 was performed. Both buffers (buffer III from our study and the buffer COMAV from López et al., 2015) contain PVP, while buffer I does not, suggesting that the presence of PVP may play a main role in infectivity after mechanical transmission of ToLCNDV. PVP is a synthetic polymer that binds to polyphenols and minimizes polyphenol oxidase activity, avoiding

oxidation and therefore maintaining the stability of the virus during the maceration (Dijktra and Jager 1998).

Regarding buffer II (Sohrab et al. 2013), the results were poor and few plants displayed symptoms, with a maximum value of 26.7% of infected plants at 14 DPI (Table 1). In consequence, mean symptom values were lower than those of the two other buffers. However, the plants inoculated with buffer II that got infected developed very severe symptoms, reaching a symptom score of 4 at 28 DPI (Table 1). Buffer II is identical to buffer I in composition but lacks β-mercaptoethanol. This suggests that β-mercaptoethanol facilitates infection, probably by keeping the inoculum infectious for a longer period of time (Uzcátegui and Lastra, 1978).

Overall, the results indicate that buffers I and III are appropriate for preparing inocula for an efficient and complete ToLCNDV infection in highly susceptible zucchini plants under experimental conditions, although faster results and higher mean symptom score were obtained with the latter (Table 1). In consequence, it is recommended using buffer III for the mechanical inoculation of ToLCNDV. Buffer III was successfully used to perform inoculation in the subsequent cucumber, melon and zucchini inoculation (Experiment 2), as a high degree of infection was achieved.

Diagnosis based on symptoms in a collection of cucurbit varieties

The isolate of ToLCNDV (ND2014-1V), which was collected from severely infected zucchini plants, was successfully sap transmitted to different varieties of cucumber, melon and zucchini using the developed protocol. Successful mechanical inoculation of ToLCNDV is in agreement with several previous studies (Chang et al., 2010; Sohrab et

al., 2013; López et al., 2015; Sáez et al., 2016), which showed that this virus could be mechanically transmitted to cucurbit crops, although the percentage of infection success from the different studies was dependent on the inoculation buffer used.

We inoculated six cucumber, six melon and seven zucchini varieties to assess the reaction of different crops and varieties to ToLCNDV infection. None of the varieties evaluated was resistant to ToLCNDV, as most of the plants developed symptoms of infection; all melon and zucchini varieties displayed viral symptoms, and only two varieties of cucumber had less than 100% symptomatic plants at 28 DPI (62.5 and 77.8 in C5 and C6 varieties, respectively) (Table 2). In contrast, mock-inoculated controls did not exhibit any symptoms. Mean symptom score values were high for melon and zucchini, but much lower for cucumber, with a maximum average value of 1.33 at 21 DPI for cucumber, whereas for melon and zucchini, the minimum average values were 2.59 and 2.65 at 7 DPI, respectively (Table 2). Our results are in agreement with previous reports that found that melon and zucchini are more susceptible to ToLCNDV than cucumber (López et al., 2015). The percentages of symptomatic melon and zucchini plants were higher than 65.0% and the average symptom score values were close to 2.5 at 7 DPI, and almost reached the maximum of 4 at 28 DPI with 100% symptomatic plants (Table 2). Although no resistance was found in the three crops, some differences in degree of susceptibility have occasionally been found among accessions of melon and cucumber, especially in exotic materials (López et al., 2015; Sáez et al., 2016). In our case, all materials correspond to old and popular varieties in Europe, which proved to be very susceptible to ToLCNDV. Previous studies (López et al., 2015; Sáez et al., 2016) have shown that the most promising materials that could be used as sources for resistance or tolerance in melon have been found in oriental varieties, while for zucchini no promising materials have been found so far. Regarding cucumber, the lowest percentage of

symptomatic plants and mean symptom score values at the end of the assay (28 DPI) were found in cucumber variety C6, suggesting that this variety may be of interest when there is a risk of infection by ToLCNDV.

The combination of variety Z6 and inoculation buffer III was used in Experiments 1 and 2, resulting in high percentages of symptomatic plants and mean symptom scores in both experiments (Tables 1 and 2). These data, together with other evidence from our experiments, indicate that the evaluation of symptoms after mechanical inoculation at 14 and 28 DPI with an appropriate methodology is an efficient method for evaluating resistance to ToLCNDV in germplasm collections of melon and zucchini. This is in agreement with the results of López et al. (2015). For cucumber, given its lower degree of infection and milder symptoms, inoculation using viruliferous whiteflies may be more appropriate for checking tolerance or resistance, as the method bears more resemblance to natural infection conditions (Sáez et al., 2016). Additionally, given that cucumber does not always display symptoms, the use of clonal replicas or the use of progeny tests may be needed for screening individual genotypes of segregating populations in breeding programmes. However, symptomatology alone may not be appropriate to evaluate infection by ToLCNDV in cucurbit plants in commercial or experimental fields, as symptoms may be caused by other diseases or the virus may not be randomly distributed (Soler et al., 2011).

Diagnosis based on serological and molecular techniques in a collection of cucurbit varieties

The serology tests showed important differences among crops, as well as differences among the antisera tested (against ToLCNDV, TYLCV, SLCV and WmCSV), in the percentage of plants that were positive in the ELISA tests. The lowest values of ELISA positive plants were found in cucumber, with average percentages of positive plants at either 14 or 28 DPI ranging between 20.4% for SLCV and 65.7% for TYLCV (Table 3). However, for melon and zucchini, much higher values were obtained for all antisera (Table 3). In melon, the average percentage of plants testing positive ranged from 83.6% for SLCV to 92.4% for TYLCV, while in zucchini, it ranged from 90.3% for TYLCV to 96.6% for WmCSV. In all crops, the antisera that provided better combinations of high percentages of positive plants and less relative variation between 14 and 28 DPI were those produced against ToLCNDV and WmCSV, particularly the latter (Table 4). With the exception of two varieties of cucumber (C3 and C6), one of melon (M4) and one of zucchini (Z5), the percentages of positive plants with the WmCSV antiserum was always higher than or equal to those obtained with the ToLCNDV antiserum (Table 3). This suggests that the WmCSV antiserum may be the most appropriate to use, at least in detecting the isolate we assessed. The results obtained with TYLCV antiserum were more irregular, as the percentages of cucumber plants testing positive at 14 DPI were much higher than those at 28 DPI, while the contrary occurred for zucchini (Table 4).

Based on the serology tests results we suggest the use of WmCSV antiserum for the efficient detection of ToLCNDV in melon and zucchini, although some plants in an individual susceptible accession may test negative. This has to be taken into account when using this method in populations screenings where all individuals are genetically distinct. For cucumber, the serology results are less promising. Less than 70% detection was obtained with any of the four antisera tested. This suggests that other methodologies may be needed for an efficient detection of the virus in this crop. The lower viral concentration

in cucumber probably contributes to negative results even in infected plants (Sohrab et al., 2013).

The three buffers and DNA extract used for molecular hybridization gave different results depending on the crop evaluated. While in cucumber, important differences were found among the buffers, for melon and zucchini, the three buffers gave similar results (Table 3). As occurred for the serology tests, the lowest percentages of plants that were positive in molecular hybridization were found in cucumber, with average values of positive plants ranging from 61.3% for buffer A (Loewe III extraction buffer) to 78.5% for the DNA extract (Table 3). For melon and zucchini, the percentages of plants that tested positive are much higher, with average values for positive plants always above 80%. In both melon and zucchini, the percentage of plants testing positive with the DNA extract was similar to the one obtained with the three buffers (Table 3). In contrast to cucumber, in general, for melon and zucchini, the variation in the percentage of plants testing positive for molecular hybridization at 14 DPI vs. 28 DPI is small for the three buffers (Table 4), which may suggest that viral concentration from 14 DPI to 28 DPI is quite stable in these two crops. The molecular hybridization tests reveal that molecular hybridization, like serology, is efficient for the detection of ToLCNDV in melon and zucchini, with values above 85% (Table 3). In the case of cucumber, the efficiency of detection was lower than in melon and zucchini, although the best results, obtained with the DNA extract (78.5%), were better than those obtained with serology tests (with a maximum of 65.7% for the TYLCV antiserum) (Table 3). In consequence, when using DNA extract for molecular hybridization in cucumber, the results are better than the extracts with extraction buffers in ground tissue. For melon and zucchini, the DNA extract yields results similar to those obtained with extraction buffers.

The PCR test was the least variable technique among crops, and resulted in more than 92% of positive plants for the three crops (Table 3). In cucumber and melon, three out of the six accessions (i.e., 50%), and in zucchini, six out of the seven accessions (i.e., 85.7%), had 100% of the plants testing positive against ToLCNDV. The lowest values were 77.8% of positive plants in two accessions (one in cucumber and one in melon). In addition, few differences were observed between testing at 14 DPI or 28 DPI (Table 4), revealing that this is a robust method for detection of infection by ToLCNDV. Overall, the results indicate that PCR is the most efficient method for detecting ToLCNDV, even in crops such as cucumber, where the percentages of positive results are much lower when using serology and molecular hybridization. The fact that PCR is able to amplify low amounts of DNA probably contributes to its greater efficiency to confirm the presence of the virus (Hussain et al., 2000). Moreover, PCR has been demonstrated to be a more sensitive technique, usually 100 times more sensitive for plant virus detection than ELISA and molecular hybridization (Alfaro-Fernández et al. 2009, 2016).

Overall, our results show that when considering the best serology (WmCSV) and molecular hybridization (using DNA extract) detection methods, as well as when using PCR detection, the results of the diagnostic methods and the symptoms are highly correlated, at least in young plants. Together with the high percentage of plants testing positive in melon and zucchini, this indicates that these methods are highly reliable for detection of ToLCNDV in these two crops.

Conclusions

The sap mechanical inoculation method that we developed for ToLCNDV, where the virus inoculum is kept in the PVP-containing buffer III, has proven to be highly efficient in cucurbit crops. The evaluated commercial varieties of cucumber, melon and zucchini were susceptible to infection, with the most severe symptoms observed in melon and zucchini. This is additional evidence that exotic sources of variation may be needed for breeding ToLCNDV-tolerant cucurbit varieties. PCR detection was the most efficient method for detecting infection by ToLCNDV in the three crops. However, for melon and zucchini, serology tests and molecular hybridization methods also had a high degree of efficiency in detecting infected plants. The best serology results were obtained with an antiserum against WmCSV. For molecular hybridization, the best results were with DNA extract. These results provide important information on the successful sap mechanical inoculation of ToLCNDV and its efficient detection. This is important for screening collections of germplasm and for selecting tolerant or resistant materials in breeding programmes.

Conflict of interest

The authors declare no conflict of interest.

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Table 1 Incidence and severity of symptoms caused by ToLCNDV in zucchini plants (variety Z6) after mechanical inoculation with different inoculation buffers (I, II, and III).

Symptom score[a]	Inoculation buffer I				Inoculation buffer II				Inoculation buffer III			
	7 DPI[b]	14 DPI	21 DPI	28 DPI	7 DPI	14 DPI	21 DPI	28 DPI	7 DPI	14 DPI	21 DPI	28 DPI
0	9	7	2	0	13	11	11	11	4	2	0	0
1	2	1	2	1	0	0	0	0	2	2	3	0
2	3	2	5	2	1	1	0	0	6	4	3	1
3	1	2	3	4	1	2	2	0	3	5	4	5
4	0	3	3	8	0	1	2	4	0	2	5	9
Symptomatic plants (%)	40.0	53.3	86.7	100.0	13.3	26.7	26.7	26.7	73.3	86.7	100.0	100.0
Mean symptom score	0.73	1.53	2.20	3.27	0.33	0.80	0.93	1.07	1.53	2.20	2.73	3.53

[a]Symptom score values: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms.

[b]Incidence and severity assessments at 7, 14, 21 and 28 days post-inoculation (DPI). Fifteen plants were evaluated for each inoculation buffer treatment.

Table 2 Incidence and severity of symptoms caused by ToLCNDV on cucumber, melon and zucchini varieties mechanically inoculated with inoculation buffer III.

Variety	Number of inoculated plants	Symptomatic plants (%)				Mean symptoms score[a]			
		7 DPI[b]	14 DPI	21 DPI	28 DPI	7 DPI	14 DPI	21 DPI	28 DPI
Cucumber									
C1	9	33.3	77.8	100.0	100.0	0.33	0.89	1.00	1.11
C2	9	33.3	77.8	100.0	100.0	0.33	0.78	1.11	1.22
C3	9	0.0	100.0	100.0	100.0	0.00	1.00	1.00	1.33
C4	9	33.3	100.0	100.0	100.0	0.33	1.89	2.11	1.00
C5	9	44.4	66.7	66.7	77.8	0.44	1.11	1.11	0.89
C6	8	12.5	87.5	75.0	62.5	0.13	0.88	1.63	0.63
Mean		26.1±6.7	85.0±5.5	90.3±6.2	90.1±6.6	0.26±0.07	1.09±0.17	1.33±0.18	1.03±0.10
Melon									

M1	8	87.5	100.0	100.0	100.0	2.13	3.13	3.63	3.63
M2	8	82.5	100.0	100.0	100.0	2.00	3.38	3.75	4.00
M3	9	77.8	88.9	100.0	100.0	1.89	3.67	4.00	4.00
M4	9	88.9	100.0	100.0	100.0	2.89	4.00	4.00	4.00
M5	9	88.9	100.0	100.0	100.0	3.11	4.00	4.00	4.00
M6	8	100.0	100.0	100.0	100.0	3.50	4.00	4.00	4.00
Mean		87.6±3.1	98.2±1.9	100.0±0.0	100.0±0.0	2.59±0.27	3.69±0.15	3.90±0.07	3.94±0.06
Zucchini									
Z1	9	100.0	100.0	100.0	100.0	2.44	4.00	4.00	4.00
Z2	9	100.0	100.0	100.0	100.0	2.56	4.00	4.00	4.00
Z3	4	100.0	100.0	100.0	100.0	3.25	3.50	4.00	4.00
Z4	8	75.0	87.5	100.0	100.0	2.63	3.50	3.63	3.63
Z5	9	100.0	100.0	100.0	100.0	3.11	3.56	4.00	4.00
Z6	9	66.7	66.7	100.0	100.0	1.89	2.00	4.00	4.00
Z7	9	77.8	100.0	100.0	100.0	2.67	3.00	3.33	4.00

Mean	88.5±5.6	93.5±4.8	100.0±0.0	100.0±0.0	2.65±0.17	3.37±0.26	3.85±0.10	3.95±0.05

[a]Symptom score values: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, severe symptoms; 4, very severe symptoms.

[b]Incidence and severity assessments at 7, 14, 21 and 28 days post-inoculation (DPI).

Table 3 Percentage of plants that tested positive in the serological and molecular tests for cucumber, melon and zucchini varieties studied during the assay. For each of the three crops the average ±SE are included.

Variety	Number of inoculated plants	Serology tests				Molecular hybridization[a]				
		ToLCNDV	TYLCV	SLCV	WmCSV	Buffer A	Buffer B	Buffer C	DNA extract	PCR
Cucumber										
C1	9	88.9	66.7	55.6	100.0	100.0	100.0	100.0	100.0	100.0
C2	9	44.4	77.8	11.1	55.6	33.3	55.6	66.7	88.9	100.0
C3	9	77.8	55.6	11.1	55.6	77.8	66.7	77.8	88.9	88.9
C4	9	44.4	66.7	11.1	77.8	77.8	66.7	77.8	77.8	88.9
C5	9	66.7	77.8	33.3	77.8	66.7	77.8	77.8	77.8	77.8
C6	8	50.0	50.0	0.0	25.0	12.5	12.5	12.5	37.5	100.0
Mean		62.0±7.6	65.7±4.6	20.4±8.3	65.3±10.5	61.3±13.2	63.2±11.9	68.8±12.1	78.5±8.9	92.6±3.7
Melon										
M1	8	75.0	75.0	62.5	87.5	50.0	75.0	62.5	62.5	87.5

M2	8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
M3	9	88.9	88.9	88.9	100.0	100.0	88.9	100.0	100.0	100.0
M4	9	88.9	88.9	77.8	77.8	77.8	77.8	77.8	77.8	77.8
M5	9	88.9	88.9	88.9	88.9	88.9	88.9	100.0	88.9	88.9
M6	8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Mean		90.3±3.8	90.3±3.8	86.3±5.9	92.4±3.8	86.1±8.1	88.4±4.3	90.0±6.6	88.2±6.3	92.4±3.8
Zucchini										
Z1	9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Z2	9	88.9	88.9	88.9	100.0	88.9	88.9	100.0	100.0	100.0
Z3	4	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Z4	8	87.5	87.5	87.5	87.5	100.0	100.0	75.0	87.5	100.0
Z5	9	100.0	100.0	88.9	88.9	88.9	88.9	100.0	100.0	100.0
Z6	9	88.9	77.8	100.0	100.0	100.0	88.9	100.0	100.0	100.0
Z7	9	77.8	77.8	88.9	100.0	88.9	88.9	77.8	88.9	88.9
Mean		91.9±3.2	90.3±3.8	93.5±2.3	96.6±2.2	95.2±2.2	93.7±2.2	93.3±4.4	96.6±2.2	98.4±1.6

[a]The preparation and composition of the different molecular hybridization buffers and DNA extract are detailed in the Material and Methods section.

Table 4 Increases (positive values) or decreases (negative values) in the percentage of plants of cucumber, melon and zucchini that tested positive at 14 DPI to 28 DPI using serology, molecular hybridization and PCR tests. Values represent the mean of the different varieties studied for each crop (n=6 for cucumber and melon and n=7 for zucchini) ± SE.

Diagnostic technique	Variation (%) from 14 DPI to 28 DPI in positive tests		
	Cucumber	Melon	Zucchini
Serology tests			
ToLCNDV	19.2±8.7	-0.2±3.1	-10.1±5.6
TYLCV	-33.6±9.0	9.3±10.0	-41.3±13.6
SLCV	-11-1±5.0	20.1±9.5	4.2±8.8
WmCSV	-5.8±9.0	7.4±11.2	-9.7±3.8
Molecular hybridization tests[a]			
Buffer A	-9.1±13.4	7.6±3.8	1.6±3.8
Buffer B	11.4±2.9	-1.9±1.9	-3.8±6.3
Buffer C	20.4±8.8	-1.6±6.5	6.3±4.1
DNA	-1.6±7.9	0.2±6.8	3.2±4.0
PCR test	-7.9±5.8	7.4±11.1	-5.4±6.8

[a]The preparation and composition of the different molecular hybridization buffers and DNA extract are detailed in the Material and Methods section.