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Diagnosis and distribution of *Citrus tristeza virus* in northern Veracruz, Mexico

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ABSTRACT

Objective: of the research was to know the incidence of CTV to try to associate it with the yellowing and death of citrus trees.

Design/Methodology/Approach: the presence of the virus was diagnosed in seven citrus-producing municipalities in northern Veracruz. A total of 804 samples from citrus trees were collected in 90 locations belonging to the municipalities of Álamo, Castillo de Teayo, Cazones, Chicontepec, Ixhuatlán, Papantla and Tihuatlán.

Results: out of all the samples, 380 were positive for CTV; 68% corresponded to attenuated variants and 40% to severe variants. The following symptoms were observed in all the municipalities: death of branches (68%), yellowing of shoots (41%), trees with small leaves (38%), and debarking of the trunk (32%); the incidence of small fruits was 31%, and finally, generalized yellowing (19%).

Limitations/Implications of the study: to manage the disease there are various alternatives, the most frequent is the use of tolerant rootstocks, however, with the existence of severe variants there may be tree deaths even with tolerant rootstocks, so it is necessary to search for and implement other far-reaching options.

Findings/Conclusions: the results show that even with the regulations for the production and mobilization of plants, the virus is widely distributed in the seven municipalities of northern Veracruz.

Keywords: citrus, incidence, variants, virus.

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INTRODUCTION

Citrus trees can be affected by different pathogens such as bacteria, fungi, viruses, and viroids which limit the production of citrus plantations (Bar-Joseph *et al.*, 2010). The disease known as citrus decline virus is caused by the *Citrus tristeza virus* (CTV), which has been present in Mexico since 1983 when the first positive plants were detected in Tamaulipas, Veracruz (1986 and 1992), and then in Yucatán, Quintana Roo, Campeche, Morelos, Michoacán (2000), Colima and Nuevo León (2005), with severe variants in some states (Loeza-Kuk *et al.*, 2008; Herrera-Isidrón *et al.*, 2009; Rivas-Valencia *et al.*, 2017). In 2001, the NOM-031-FITO-2000 norm was published, which established "the campaign against the citrus decline virus", although the dispersion of the disease still continues (Rivas-Valencia *et al.*, 2010; Contreras-Maya and Villegas-Monter, 2020).

Citrus decline has been one of the most devastating diseases in the citrus producing zones of the world; in the 1930s and 1940s it affected practically the entire citrus production in Argentina, Brazil and Uruguay, and by 1950 in Colombia and Peru, which had rootstock of sour orange (*Citrus aurantium*), and as a result 20 000 000 trees were eliminated (Müller and Rezende, 2004). In 1980, another similar disaster happened in Venezuela and Jamaica (Mendt, 1992; Roistacher, 1999). However, it is unknown why such an epidemic had not happened in Mexico, considering that since 2003 there are reports of severe isolates in *C. sinensis* in the states of Baja California, Nuevo León, Tamaulipas and Veracruz (Herrera-Isidrón *et al.*, 2009; Mendoza *et al.*, 2003; Loeza-Kuk *et al.*, 2005; Contreras-Maya and Villegas-Monter, 2020; Contreras-Maya *et al.*, 2022). There are several isolates of CTV that differ biologically and primarily in the symptoms that they cause in citruses (Dawson *et al.*, 2013, 2015). The variants responsible for the sudden decline have been designated as severe, while the ones that do not cause symptoms are known as mild. The CTV is transmitted through infected shoots and in a semi-persistent way by many species of aphids (Harper *et al.*, 2016).

In northern Veracruz, in recent years, symptoms of yellowing, dry branches, small leaves and fruits, and even tree deaths have been seen, which lead to the deterioration and low yield of plantations. There is lack of knowledge of the pathogens associated to yellowing. Therefore, the aim is to evaluate the presence and distribution of CTV and its variants in seven orange producing municipalities in northern Veracruz, Mexico, with the aim of updating the phytosanitary status of CTV and to give it the importance it has as a threat to Mexican citrus production, which could be devastating as it has happened in other countries, since the sour orange rootstock predominates.

MATERIALS AND METHODS

Sample collection

The study was conducted in citrus plantations located in the municipalities of Álamo (153 samples), Castillo de Teayo (76), Cazones (117), Chicontepec (100), Ixhuatán (79), Papantla (183), and Tihuatlán (96), which are the leading orange producers in northern Veracruz, where 804 citrus samples were collected in 90 localities. The sampling was directed at trees with symptoms of yellow shoots, death of branches, small fruits, trunk debarking, and small leaves. Four vegetative shoots were collected in each tree in active growth (a shoot per cardinal orientation), which were labeled and placed in polyethylene bags and stored in cold, to transport to the Biotechnology and Plant Physiology Laboratory, Montecillo Campus, Colegio de Postgraduados. In addition, a questionnaire was carried out with the producers to understand the origin of the plants, as well as the age, variety, rootstock and management.

RNA extraction and molecular analysis

Once the samples arrived to the laboratory, they were stored at −4°C for their processing, which consisted in cleaning the leaves with interfolded paper towels (Sanitas®, Mexico) and alcohol at 75%, then the leaf midribs were extracted with single-edge razors (CORTY®, Mexico) and finely chopped for storage in microtubes of 2 ml (AXYGEN®,

Mexico) at −20 °C. Tissue maceration was done in a disruptor (Retsch®, Mexico) at 30 frequencies per 20 min, with 0.25 g of leaf midrib plus 1 ml of saline buffer (Tris-HCL 10mM, EDTA 1mM, NaCl 2M, Bovine albumin 0.05%; Sigma-Aldrich, USA) and 3 stainless steel pellets (3/16") in microtubes of 2 ml. Once the tissue is macerated, the microtubes were centrifuged for 5 minutes at 13500 rpm at 4 °C; the pellets were retrieved and the supernatant discarded; again, 800 μL of saline buffer were added, there was vortex and then it was centrifuged for 5 minutes at 13500 rpm at 4 °C and the supernatant discarded.

For the extraction of nucleic acids, the Minas *et al.* (2011) protocol was followed with some modifications. In the phase of cell lysis, CTAB 2% was used (Tris-HCL 1M (pH 8; Sigma-Aldrich, USA), EDTA 20 mM (pH 8; Sigma-Aldrich, USA), NaCl 1.4 M (pH 8; Sigma-Aldrich, USA), and CTB 2% (w/v; Sigma-Aldrich, USA)), vortex happened. All the samples were incubated at 65 °C for 40 minutes, centrifuged at 13500 rpm 4 °C for 5 min, 800 μL of the supernatant was transferred to microtubes of 2 ml adding 400 μL of phenol: chloroform: isoamyl alcohol (25:24:1; invitrogen, USA) to each sample, it was mixed by inversion during 3 min at room temperature. During this phase of enzyme inhibition and precipitation of nucleic acids, the microtubes were centrifuged at 14000 rpm for 10 min at 4 °C to recover 500 μL of the supernatant in a microtube of 1.5 ml, adding 500 μL of isopropyl alcohol (−20 °C) and 50 μL of ammonium acetate 7.5 M (CH_3COONH_4; Sigma-Aldrich, USA), and it is left to incubate all night at −20 °C. The samples were centrifuged at 14000 rpm for 10 min at 4 °C, and the supernatant was discarded. Finally, the pellets or pills were washed with ethanol (Sigma-Aldrich, USA) at 70% (v/v) 1 ml at 14000 rpm for 5 min at 4 °C; the pellets were left to dry at room temperature and were re-suspended in 50 μL of water free of nucleases (IDT, USA), stored at 4 °C for 24 h.

Then, the qualities (260/280 nm; 260/230 nm) and RNA concentrations were verified through spectrophotometry using the Nano Drop 2000 (Thermo Fisher Scientific, USA) with 1 μL of total RNA sample.

In the reverse transcription reaction (RT; Reverse Transcription), each microtube of 0.2 ml was added with 0.75 μL of each primer (P25-R, VT-R, T30-R at 10 pmol, Table 1) in 6.87 μL of water + 3 μL of RNA from each sample and incubated at 72 °C for 5 min in a thermocycler (MAXIGENE AXIGEN, USA), unique cycle. Then, each sample was added with 7.87 μL of the mixture that contained 5 μL Buffer 5X of M-MLV + 1.5 μL of dNTP's Mix + 0.625 μL of RNAsin + 0.75 μL of M-MLV Reverse Transcriptase (Promega, USA), placing the tubes in the thermocycler at 42 °C for 60 min followed by 72 °C for 10 min, unique cycle.

The samples were analyzed through qPCR for the detection of CTV in a thermocycler C1000 (Bio-Rad, USA). The final reaction volume was 20 μL: 10 μL of Ssoadvance universal for sybr green mix (Bio-Rad, USA), 0.5 μL of each primer P25F-23 and P25R-20 (125 nM) (Saponari *et al.*, 2008), 7 μL of water free of nucleases and 2 μL of cDNA. Each sheet contained two Not target and NTC control which validated the absence of contaminants or dimers in the reaction; they were fit with the adhesive cap

manipulating it only by the edges. The thermocycling program was 55 °C for 2 min with initial denaturalization followed by 95 °C for 5 min, then 40 cycles at 95 °C for 15 s, and aligning at 57 °C for 40 s. Finally, a melting curve of 65 to 95 °C was used with increase of 0.5 °C/cycle.

Considering the viral concentration, samples were selected for the final point PCR reaction, with the objective of identifying existing severe and attenuated variants, using for this purpose a thermocycler (MAXIGENE AXIGEN, USA). It was done by adding to each tube 9 μL of the mixture that contained 2 μL Green buffer GoTaq DNA Polymerase + 0.4 μL of $MgCl_2$ (10 Mm) + 0.2 μL of dNTP's (10 Mm) + 0.6 μL of each primer (VT-F, VT-R, T30-F and T30-R) + 0.1 μL of GoTaq DNA Polymerase (Promega, USA) + 2 μL of cDNA + 5.1 μL of water. The PCR conditions for the five variants were: one cycle of 3 min 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 56 °C, 45 s at 72 °C and a final extension of 10 min at 72 °C (Saponari *et al.*, 2008; Contreras-Maya and Villegas-Monter, 2020; Contreras-Maya *et al.*, 2022). The products were visualized in non-denaturalizing agarose gel at 2%, dyed with ethidium bromide. The gel was observed and photographed in a QUANTUM ST5® (Vilver Lourmat, USA) transilluminator, using positive, negative and 100bp DNA Ladder controls (Promega, USA). Some of the samples that were positive were sent to Macrogen Corp. (South Korea) for sequencing and thus corroborate the presence of CTV.

Impact of symptoms and CTV

According to the producers and the field visits that were carried out, the symptoms were grouped into: generalized yellowing, yellow shoots, death of branches, small fruits, trunk debarking, and small leaves. Therefore, the sampling was directed at trees that presented one or more of the symptoms mentioned before; 804 samples were collected, among which 722 were orange trees (*Citrus sinensis* L. Osbeck), species that predominates in northern Veracruz, 33 Persian lime (*Citrus latifolia* Tanaka), 31 mandarin (*Citrus reticulata* Blanco), and 18 grapefruit (*Citrus paradisi* Macf.). To determine the impact of each of these symptoms, the number of plants with symptoms was divided by the total number of trees collected by municipality and multiplied by 100. In the case of the impact of CTV, the number of samples that were positive in the qPCR reaction was also taken into account, as well as the total number of samples by municipality.

Table 1. Sequences of primers for RTqPCR and RT-PCR.

Name	Polarity	Sequence 5´-3´	Size (pb)	Product size	Reference
P25-F	Sense	AGCAGTTAAGAGTTCATCATTAC	23	101	Saponari *et al.*, 2008
P25-R	Antisense	TCAGTCCAAAGTTTGTCAGA	20		
VT-F	Sense	TTTGAAAATGGTGATGATTTCGCCGTCA	28	302	Roy *et al.*, 2010
VT-R	Antisense	GACACCGGAACTGCYTGAACAGAAT	25		
T30-F	Sense	TGTTGCGAAACTAGTTGACCCTACTG	26	206	
T30-R	Antisense	TAGTGGGCAGAGTGCCAAAAGAGAT	25		

RESULTS AND DISCUSSION

The RT-qPCR technique allowed detecting the pathogen in 380 positive samples to the citrus decline virus. The primers P25F-23 and P25R-20 amplified for the protein layer of the virus. The samples that amplified with Cq (Cycle quantification) <35 were considered positive and presented temperature or melting curve (fusion curve) Tm=80.5 °C (±0.5 °C) that corresponded to the product obtained by the primers, and in addition, the NTC did not present amplifications, which confirmed the absence of primer dimers or of any type of non-specificity (Figure 1).

Distribution of the virus

Mexico is considered one of the leading citruses producing countries; however, in recent years the yield has decreased due to different biotic and abiotic factors (Orozco-Santos *et al.*, 2014). Among these, we can highlight problems with diseases such as the one caused by the citrus decline citrus, whose impact varied from 27% in Chicontepec to 63%

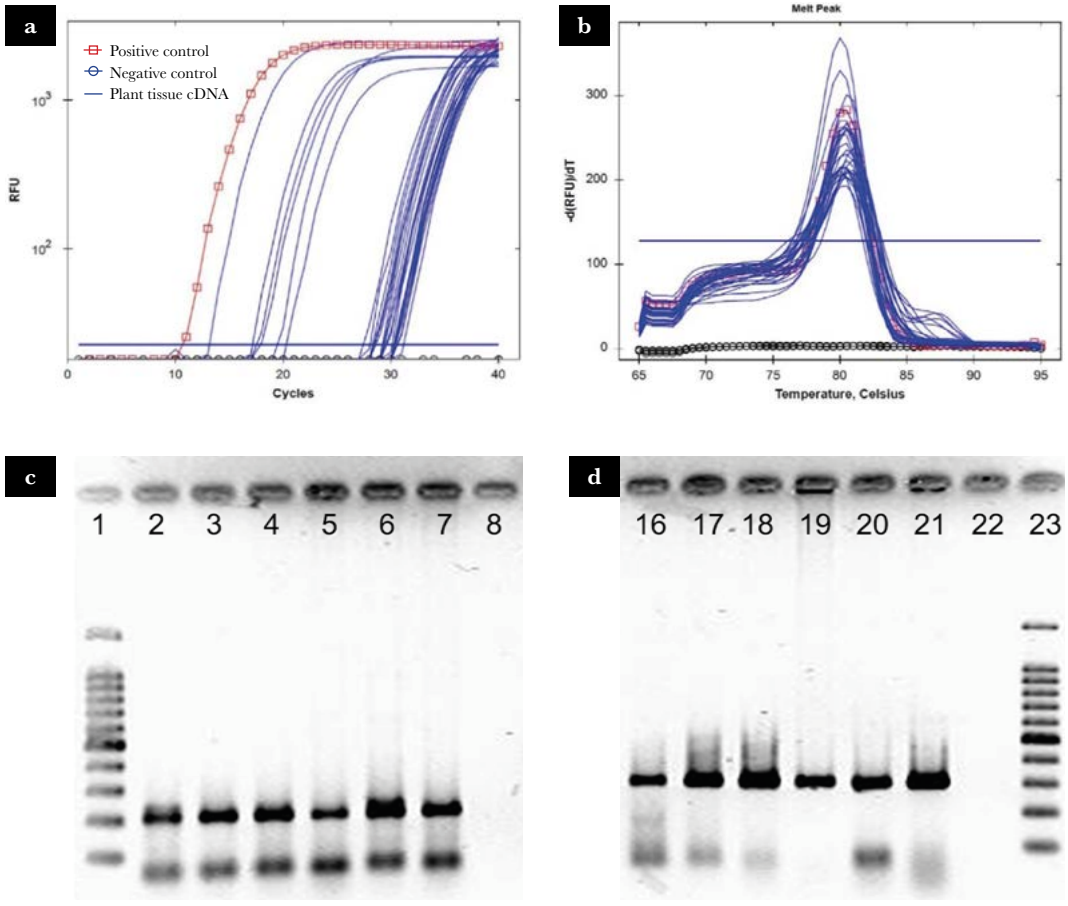

Figure 1. Amplification by qPCR of *Citrus tristeza virus* in samples of symptomatic plant tissue. a) Amplification curves obtained from cDNA; b) Fusion curves for CTV amplification, the temperature (°C) is presented on the (x) axis, and the fluorescence units (RFU) on the (y) axis; c) Amplifications obtained by final point RT-PCR for attenuated variants (T30); and d) Amplifications for severe variants (VT) of CTV, where lanes 1 and 23 correspond to the molecular weight marker of 100 pb; 2 and 16 positive controls; lanes 8 and 22 negative controls; lastly, 3-7 and 17-21 samples of citruses that were positive.

in Álamo. In Álamo and Papantla, more than 50% of the samples were positive, situation that is worrying because of the distribution of the disease since more than 50% of the surface planted with citruses in northern Veracruz is concentrated in these municipalities. It should be indicated that in Chicontepec and Ixhuatlán there were lower percentages; however, this does not exempt them from reaching risk zones because at any time the impact could increase because most of the producers use non-certified plants purchased in nurseries of the municipality of Álamo with the highest percentage of CTV according to the study's results.

Propagation of the virus and its variants

The CTV is transmitted in a semi-persistent way by *Aphis gossypii* and *Toxoptera citricida*, among other aphids (Cambra *et al.*, 2000). However, the high impact allows indicating that the virus has also been disseminated by the mobility of the infected plant material (purchase of non-certified plants), in addition to recent reports that CTV can be transmitted by diaphorina citri (Wu *et al.*, 2021; Zhang *et al.*, 2023). Taking into account that more than 95% of the plants used come from non-certified nurseries, where the plants are produced outdoor and the shoots are collected from trees of unknown origin and that the federal campaign against CTV was eliminated since 2014, it is easy to understand why there is massive death of citrus trees in northern Veracruz and there is no doubt that the same can happen in the rest of the country in a short amount of time.

The detection of severe variants of CTV in mandarin plantations in California in the year 2000 originated from the illegal imports of shoots (Matos *et al.*, 2013), the discovery of VT type variants in commercial citruses in Florida (Hilf *et al.*, 2005), which represent clear examples that severe variants can emerge due to oversight in the introduction of plant materials, which are a continuous threat for the citrus industries in many countries. The eradication of infected trees, as well as the development of means to protect the plantations against severe variants, become critical to coexist with the virus.

It should be mentioned that in Mexico, the first detections of CTV were carried out in Tamaulipas (1983) and Veracruz (1986); however, despite the eradication of the initial focal points, the dispersion of the disease has continued (Silva-Vara *et al.*, 2001; Rivas-Valencia *et al.*, 2010; Contreras-Maya *et al.*, 2022). Currently, there are several aphid species that can transmit CTV, the main ones are *Aphis gossypii* (Glover), *A. spiraecola* (Patch), *Toxoptera aurantii* (B de F) and *T. citricida* (Kirkaldy). *T. citricida* is found in all the citrus producing states of the country, with the risk of causing severe outbreaks of citrus decline because severe variants have been detected in Nuevo León, Colima, Baja California and Tamaulipas (Mendoza *et al.*, 2003; Loeza-Kuk *et al.*, 2005; Herrera-Isidrón *et al.*, 2009), and recently in several municipalities of Veracruz (Contreras-Maya *et al.*, 2022). Taking this into account, the situation is more worrying because the disease is widely distributed and there are increasingly more vectors that favor the dispersion. A factor that is not considered is the number of non-certified nurseries, which in northern Veracruz exceed 400 selling points, because they have never been controlled and are probably the most important vector.

Variants of CTV of the attenuated and severe type were found in every municipality sampled, and more than two variants were even found in the same tree. It should be pointed

out that in Álamo, 60% of the CTV-positive samples were VT type while in Castillo only 3% (Figure 2). The presence of severe variants in all the municipalities and the use of sour orange as rootstock explain the accelerated death of plants in northern Veracruz, but it is important to consider that psorosis, exocortis, and cachexia are also present based on the symptoms observed in the plant trunk and branches. These diseases have been reported previously by Almeyda *et al.* (2007) and Contreras-Maya *et al.* (2018).

Symptoms associated to citrus death

Concerning the symptoms, trees were observed with death of branches (68%) in every municipality, with higher impact in the municipalities of Tihuatlán, Papantla, Cazones, Castillo de Teayo and Álamo. Another symptom that presented high impact was yellowing of shoots (41%), principally in Tihuatlán, Papantla, Álamo and Cazones. The symptoms of small leaves and trunk debarking had values of 38 and 32%, respectively; the trees with small fruits had impact of 31%, and lastly generalized yellowing (19%) (Figure 3). However, it is difficult to attribute the death of plants to a causal agent, because the problem presents in citrus producing zones is multifactorial. Nevertheless, there are plants that die because of the presence of CTV and it can be verified in the field because the leaves and the fruits remain adhered to the plant (Figure 3e), where the classical syndrome is present, or else the rootstock dies from the base and this causes the death of the variety (Figure 3d). The producers manage to distinguish a sudden death of the trees and colloquially called it "died out of nowhere", term that is frequently heard in the community of Santa Emilia, Álamo, Veracruz.

Depending on the virus variant and the citrus graft/rootstock combination, CTV causes two primary symptoms, which have had an impact on the global production of citrus. The first is the sudden decline of trees grafted in sour orange (*Citrus aurantium*). During the past century, the severe outbreaks of CTV caused a fast decline that developed in the citrus producing regions and destroyed nearly 100 million trees (Moreno *et al.*, 2008). The solution to the outbreak was to use disease-tolerant rootstock. Although this allowed an effective control of CTV, these rootstocks require a different management than sour orange (Folimonova *et al.*, 2013), including fertilization, which is scarcely used in the study

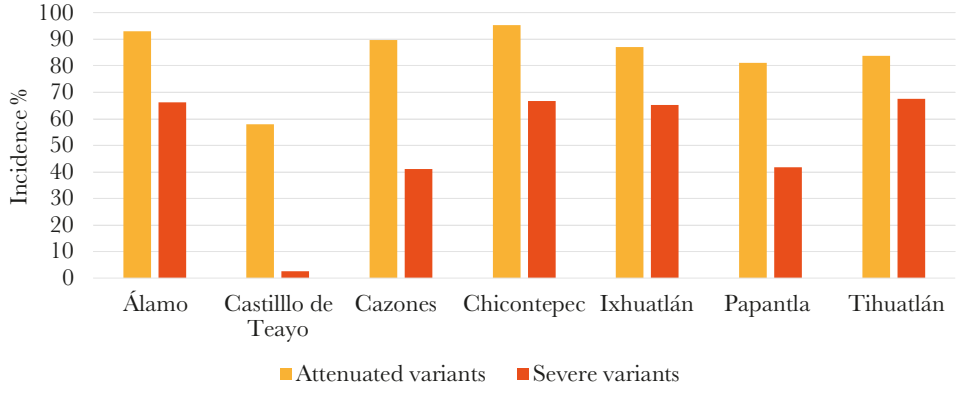

Figure 2. Impact of *Citrus tristeza virus* variants in seven municipalities of Veracruz, based on the samples collected by municipality and which were positive in the qPCR reaction.

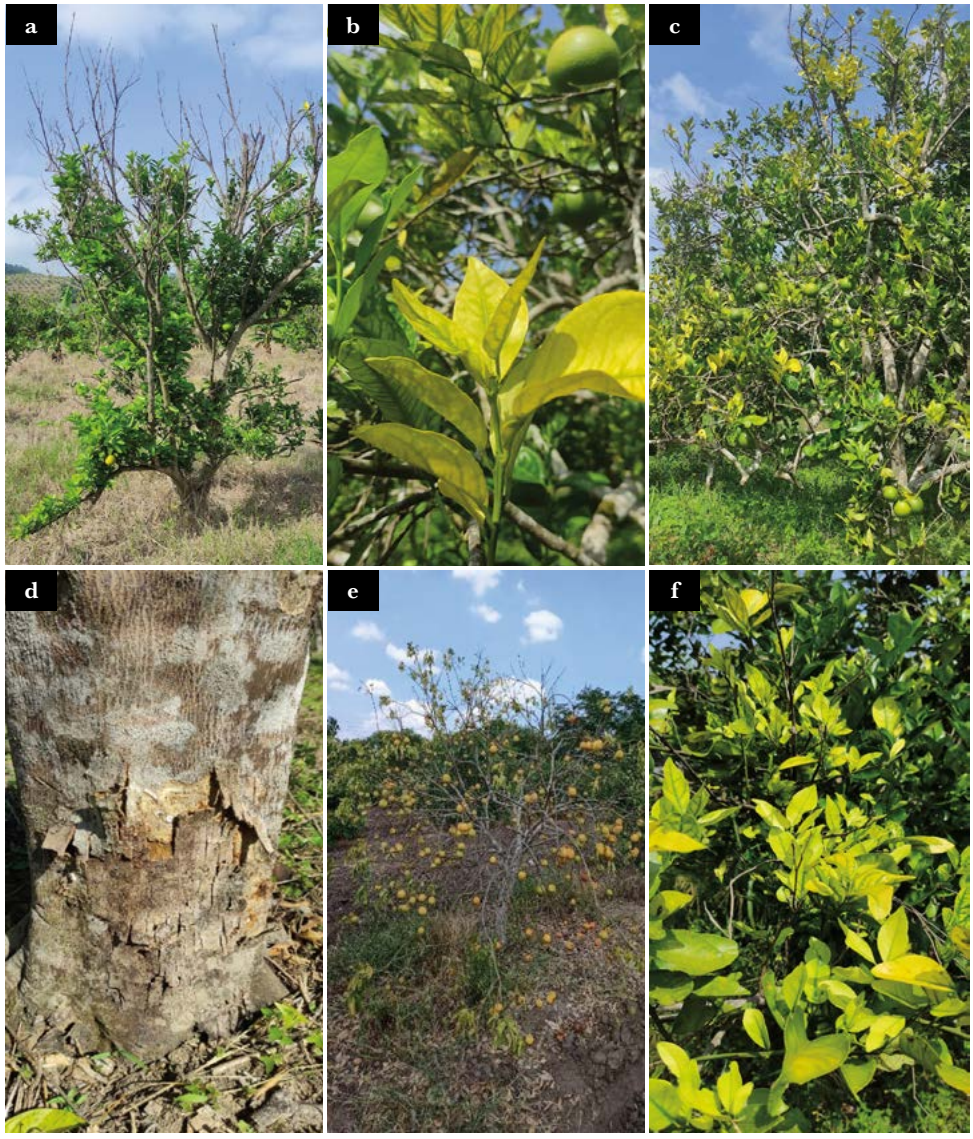

Figure 3. Symptoms observed and associated to *Citrus tristeza virus* in seven municipalities of northern Veracruz. a) Dry branches (68%), b) yellow shoots (41%), c) small leaves (38%), d) trunk debarking (32%), small fruits (31%), and generalized yellowing (19%).

zone, tree pruning, considered until now as unnecessary in citrus trees, although essential for the "new citrus production" and irrigation; due to climate change, it is necessary to apply four to six irrigation events in the months of April-May, July-August, and this if we want to continue producing citrus trees.

Another symptom caused by some variants of CTV is stem pitting, which affects grapefruit (*C. paradisi*), orange (*C. sinensis*), and Mexican key lime (*C. aurantifolia*), regardless of the rootstock used. The stem pitting is the result of the areas of virus multiplication (Tatineni *et al.*, 2011). However, during the sampling carried out, stem pitting was not observed, although rootstock death was, starting from the thinnest roots to those of first order, and then the stem base which climbs progressively; this is what we see in the field,

death from the tips of the terminal branches and the continuing descending death, which confuses technicians and producers because they do not observe the base of the trunk, in plants with these symptoms that are consistently found in VTC.

From the symptoms evaluated, death of branches, leaves with yellow midribs, small leaves and fruits are related to *Citrus tristeza virus*, which is why the cause of death can be due to this virus; however, we cannot forget that the plants also have psorosis, exocortis, cachexia, HLB, leprosis, in addition to the attack from fungi (*Colletotrichum* spp. and *Lasiodiplodia* spp.) oomycetes, lethal fungus (*Usteulinea deusta*); these pathogens weaken the plant and as consequence it is more susceptible to diseases. We should also take into account that in northern Veracruz most of the producers do not fertilize and pruning is incipient; these two factors also impact the "presence, or make the plants more sensitive to attack from diseases". With the results obtained, it is possible to point out that: there is NO control in the mobility of plants (certified material is generally not used), and there is no control of vector insects. There is lack of knowledge of the main diseases of economic importance, which evidence the lack of training of technicians and producers in general.

CONCLUSIONS

The high impact of CTV and severe type VT variants explain in part the death of plants in northern Veracruz, although we must not forget that there are other quarantine diseases that are also contributing and aggravating the problem.

Some of the samples collected were young plants, which were positive to *Citrus tristeza virus* and this shows the risk that viability and productivity present in the orange plantations in northern Veracruz.

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