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Photosynthetic response of citrus to *Citrus tristeza virus* isolates with potential for cross-protection

Abstract – The objective of this work was to evaluate the photosynthetic response of citrus varieties to the inoculation of isolates T30 and T3 of *Citrus tristeza virus* (CTV) characterized as having potential to provide cross-protection against severe CTV isolates in citrus. Citrus plants of 'Campbell' orange, 'Persian' lime, and 'Key' lime were subjected to inoculations by both isolates by patch. Noninoculated plants were used as the control. Symptom expression, photosynthetic and transpiration rates, and stomatal conductance were evaluated by IRGA. Stomatal density and size were evaluated by epidermal impressions. The CTV isolates were also evaluated and molecularly characterized by RT-PCR and Sanger sequencing. 'Campbell' orange and 'Persian' lime plants remained asymptomatic after the inoculations. The inoculation treatments with both CTV isolates did not reduce photosynthetic capacity, transpiration rate, and stomatal conductance, in comparison with the control. Stomatal density and size varied according to the citrus species. T30 and T3 do not affect the photosynthetic responses of 'Campbell' orange and 'Persian' lime; therefore, these isolates have the potential to provide cross-protection to citrus varieties against severe isolates of CTV.

Index terms: *Citrus latifolia*, *Citrus sinensis*, cross-protection, photosynthesis, stomatal density.

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Resposta fotossintética de citros a isolados de *Citrus tristeza virus* com potencial para proteção cruzada

Resumo – O objetivo deste trabalho foi avaliar a resposta fotossintética de variedades de citros submetidas à inoculação dos isolados T30 e T3 de *Citrus tristeza virus* (CTV) caracterizados como tendo potencial para conferir proteção cruzada a citros contra isolados severos de CTV. Plantas de citros de laranja 'Campbell', limão 'Tahiti' e limão 'Galego' foram submetidas à inoculação de ambos os isolados por "patch". Plantas não inoculadas foram utilizadas como controle. A expressão dos sintomas, as taxas fotossintética e de transpiração, e a condutância estomática foram avaliadas por IRGA. A densidade e o tamanho estomáticos foram avaliados por impressões epidérmicas. Os isolados de CTV também foram avaliados e caracterizados molecularmente por RT-PCR e sequenciamento de Sanger. As plantas de laranja 'Campbell' e limão 'Tahiti' permaneceram assintomáticas após as inoculações. Os tratamentos de inoculação com ambos os isolados de CTV não reduziram capacidade fotossintética, taxas de transpiração e condutância estomática, em comparação ao controle. A densidade e o tamanho estomáticos variaram de acordo com a espécie. T30 e T3 não afetam as respostas fotossintéticas de laranja 'Campbell' e limão 'Tahiti'; portanto, estes isolados têm potencial para

promover proteção cruzada a variedades de citros contra isolados severos de CTV.

Termos para indexação: *Citrus latifolia*, *Citrus sinensis*, proteção cruzada, fotossíntese, densidade estomática.

Introduction

Worldwide citrus fruit production is significant and primarily distributed in tropical and subtropical regions in more than 140 countries. Annual production exceeds 38 million tonnes; China, Brazil, India, Mexico, United States, and Spain are the top-producing countries. Mexico is the second exporter of lemon worldwide with 571,175 ha of planted citrus producing 8,548,845 tonnes (FAO, 2021). However, different pathogens affect citrus production, among which the *Citrus tristeza virus* (CTV) is the causal agent of one of the most destructive diseases. It replicates in the phloem cells in the *Citrus* genus. The symptoms depend on the host, variety, climate conditions, vector populations, and on the infecting CTV isolates (Catara et al., 2019).

CTV has killed millions of trees in Argentina, Brazil, South Africa, United States of America, and Spain, and yet, it continues to spread to new areas, either by infected buds or transmitted by different aphid species. Three specific symptoms such as decline, stem pitting (SP), and seedling yellowing (SY). As mentioned before, severity varies depending on the host, CTV isolates, and abiotic stress factors acting on the plant (Yokomi et al., 2018).

The current disease prevention includes the use of tolerant rootstocks for crop protection and, in some cases, the management of aphids, the main vectors of CTV. Crop protection is a method that reduces the damage caused by infection from severe virus isolates by pre-inoculation with mild or asymptomatic isolate of the same virus (Leonel et al., 2015). A typical CTV infection contains a mixture of genotypes with various biological properties and severity. Thus, a successful cross-protection program requires knowledge on the characteristics of local isolates. Brazil and South Africa have successfully used the cross-protection technique in commercial citrus production. Recently, the state of California, USA, has started employing this strategy. Therefore, it is foreseeable that the future citrus industry will require cross-protection strategies to deal with severe CTV isolates.

Pathogen infections generally alter the plant physiology. The photosynthesis is one of the most important physiological aspects of plants that is negatively affected by stress caused by biotic or abiotic stresses. Reduced photosynthetic activity during the pathogenic stress decreases the chlorophyll synthesis and inhibits the Calvin cycle activities directly or indirectly. Plants facing biotic stress, such as viral infection, react by producing reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), O_2- (superoxide), and OH (hydroxyl) radicals. ROS may function as a signal transduction pathway, generating oxidative damage to plant structures when their production is excessive (Hančevič et al., 2018). However, the effects of CTV infection on the photosynthetic machinery have been little studied.

Stomata are specialized leaf structures that regulate gas exchange. Two protective cells surround the pore, linking the intercellular spaces inside the leaf to the atmosphere. Carbon dioxide reaches the chloroplasts in the mesophyll through the stomata as a substrate for photosynthetic reactions (Padoan et al., 2013). In recent years, stomata have been recognized as part of the plant immune system; however, they may also act as an entry site for infections by fungi, bacteria, and nematodes. Information on viral infections and their effect on stomatal density, and, consequently, on plant metabolism, is still scarce. Murray et al. (2016) showed that inoculation with TVCV (*Turnip vein clearing virus*) in susceptible *Arabidopsis thaliana* plants reduced the stomatal density (SD) by 12.3%, whereas, in TVCV-tolerant *Chenopodium quinoa* plants, SD reduction was not significant.

The objective of this work was to evaluate the photosynthetic response of citrus varieties to the inoculation of isolates T30 and T3 of CTV characterized as having potential to provide cross-protection against severe CTV isolates in citrus.

Materials and Methods

The experiment was carried out in northern Veracruz, Mexico, in greenhouse conditions on plants subjected to inoculation of CTV isolates characterized as T30 and T3. 'Campbell' orange (*Citrus sinensis* L. Osbeck) and 'Persian' lime (*Citrus latifolia* Tanaka) grafted on Volkamerian lemon (*Citrus volkameriana* Pasq) were used in the study. Seedling 'Key' lime

(*Citrus aurantifolia* Swingle) was used as an indicator plant. Completely randomized designs were conducted with the following factorial arrangements: factor A, inoculated and noninoculated plants; factor B, three citrus species; and factor C, three different origins – T1, orange 1 Rancho Nuevo; T2, orange 2 Rancho Nuevo; T3, orange 3 Castillo de Teayo were selected as bud and bark donors for inoculation.

Firstly, donor bud sticks from molecularly identified CTV T30+T3 isolates were used according to Roy et al. (2010). Bud sticks identified as CTV-carrying type T30+T3 were grafted onto *C. volkameriana*. Their health was visually monitored every 15 days. CTV (T30+T3) presence was confirmed by Sanger sequencing one year after grafting at Humanizing Genomics Macrogen (Seoul, South Korea).

'Campbell' orange, 'Persian' lime, and 'Key' lime seedlings were subjected to the inoculation of CTV T30+T3, using patches (bark). Noninoculated plants were used as control. Five replicates by species were used. The presence of CTV presence was confirmed by PCR one month after inoculation and, subsequently, every three months for one year.

The RNA extraction was carried out following the protocol by Contreras-Maya et al. (2022). Samples were analyzed by RT-PCR with the primers T30-F, T30-R, and T3-F, T3-R. For the reverse transcription (RT) reaction, 0.5 µL of each primer (F and R) was added to each 0.2 mL microtube with 4 µL of free DNAse water and 2 µL of RNA (200 µg μL^{-1}), from each sample, and incubated at 72°C for 5 min in a thermocycler Techne TC-512. Subsequently, the microtubes were placed on ice for 10 min. For each sample, 4 µL were added to the mix containing 2 µL M-MLV 5X buffer (Promega Corporation, Madison, USA), 1 µL of 0.1mol L^{-1} DTT (Promega), 0.5 µL of dNTPs Mix (Promega), and 0.15 µL of M-MLV Reverse Transcriptase (Promega); the microtubes were incubated in a thermocycler at 42°C for 60 min followed by 10 min at 72°C. For the PCR analysis, to each 0.2 mL microtube, 9 µL were added to the mix containing 2 µL of Green buffer GoTaq DNA Polymerase (Promega), 0.4 µL $MgCl_2$, 0.2 µL of dNTPs mix, 0.6 µL of each primer (F and R), 0.1 µL of GoTaq DNA Polymerase (Promega), 5.1 µL of free DNAse water, and 2 µL of cDNA. The PCR conditions proposed by Roy et al. (2010) were followed. The PCR products were visualized on a 2% agarose gel with ethidium bromide.

The PCR products were sequenced in both directions (forward and reverse) at Humanizing Genomics Macrogen (Seoul, South Korea). The sequences were assembled and edited with BioEdit Sequence Alignment Editor v 7.2.6 software by Informer Technologies, Inc. and compared using the Basic Local Alignment Search Tool (BLAST) with those at the National Center for Biotechnology Information (NCBI, 2023).

To represent the phylogeny of C*itrus tristeza virus*, the sequences of CTV genotypes T30 and T3 were downloaded from the NCBI GenBank database (AF260651.1 and EU857538.1). All consensus sequences were compiled in FASTA format. Multiple sequence alignment (MUSCLE) was performed with the MUSCLE application, included in the Molecular Evolutionary Genetics Analysis v7 (MEGA) software (Kumar et al., 2016), where the ends of the sequences were cut. The phylogenetic analysis was performed with the neighbor-joining (NJ) method, and the nucleotide substitution model obtained was K2 (Kimura 2 parameters); a bootstrap of 500 replicates was performed.

The physiological parameters photosynthetic rate, number and size of stomata were evaluated 12 months after the inoculation. The photosynthetic rate was quantified with a portable IRGA system (LI-6400/XT, Li-cor, Lincoln, NE, USA). One leaf was analyzed from each of four randomly selected trees per treatment (four replicates). The selected leaves were similar in size, fully matured, and located on branches of the last growth. Data were subjected to the analysis of variance, and mean comparison tests were performed by Tukey's honestly significant difference (HSD), at 5% probability, using the Statistical Analysis System (SAS) software package.

The number of stomata was obtained from epidermal impressions of abaxial surfaces from fully expanded mature leaves (corresponding to the previous growth flow). Impressions were made by evenly covering the middle part of each side of the middle leaf vein, using instant cyanoacrylate glue (Industrias Kola Loka, S.A. de C.V., Mexico). The glue-covered leaf section was pressed onto slides, and one minute later, the leaf was carefully peeled off. Images were taken using a Tessovar microscope (Carl Zeiss AG, Oberkochen, Germany) fitted with a Canon EOS 100D camera and

a Canon EF-S 60 mm f/2.8 Macro USM lens (Canon Mexicana, S. de R.L. de C.V., México).

For the stomatal density (SD), which measures the number of stomata per unit of leaf area, the stomata count per field was performed with the ImageJ program under a 10X objective. Two prints were made per leaf, and one field was photographed per print. Ten fields per treatment (each one with 1.4 mm^2 area) were evaluated. Density was expressed as the number of stomata per square millimeter. Stomata length and width (µm) were measured with ImageJ on ten stomata per field (40X); 100 measurements per treatment were collected.

Results and Discussion

The presence of CTV T30+T3 viral RNA was detected. The amplification with specific primers based on the protein coat gene (P25) generated fragments of 206 bp (T30) and 409 bp (T3), identifying the T30 and T3 genotypes (Roy et al., 2010). The sequences were compared with sequence types T30 and T3 downloaded from the NCBI GenBank, the samples under study were identified with 92–99% similarity to *Citrus tristeza virus* complete genome from types T30 and T3 from the GenBank (Figure 1).

During the experiment, no disease symptoms were observed in 'Campbell' orange and 'Persian' lime, whereas 'Key' lime plants showed slight yellowing of leaf veins (Figure 2). The biological indexing is indispensable to evaluate CTV isolates with potential pre-immunization (Atta et al., 2017). CTV genotypes that could serve for cross-protection do not cause symptoms in *C. sinensis* and cause only slight yellowing in *C. aurantifolia*, according to Leonel et al. (2015). In a study characterizing the biology of CTV genotypes, Besoain et al. (2015) found that some attenuated isolates caused only yellowing of the veins of 'Key' lime plants.

The inoculations of T30+T3 isolates did not significantly affect the net photosynthesis rate, but they significantly affected conductance and transpiration (Figure 3). There was a significant effect for species, for which 'Key' lime showed higher photosynthesis rate, conductance, and transpiration values than those of 'Campbell' orange and 'Persian' lime.

'Key' lime showed a higher CO_2 assimilation rate (9.59 µmol CO_2 m^{-2} s^{-1}) than that of 'Campbell' orange (7.63 µmol CO_2 m^{-2} s^{-1}). Ribeiro et al. (2003) mentioned that in sweet orange plants infected by *Xylella fastidiosa*, leaf water potential, CO_2 assimilation, transpiration, and stomatal conductance tended to decrease. However, in the present study, the opposite trend was observed.

Net photosynthesis was observed to decrease by virus infection; additionally, reductions of light-dependent processes, stomatal conductance, or activity of carbon fixation reactions of the Calvin cycle have been associated (Souza et al., 2017). However, decreases of photosynthesis values can vary with species, cultivar, and virus genotype. Some CTV genotypes cause visible changes of leaf coloration and turgor, making it reasonable to assume that there is a reduction of virus-induced photosynthesis, which might be associated with a reduction of stomatal conductance and possibly of the activity of reaction in thylakoids. In the present study, CTV increased the photosynthesis, conductance, and transpiration.

The stomatal conductance and transpiration rate in 'Key' lime and 'Campbell' orange plants increased, while in 'Persian' lime, these parameters decreased (Figure 3), and the stomatal conductance averages in the 'Key' lime plants were higher than those of orange and 'Persian' lime. In contrast, in the control plants, the values were lower in the three species.

Salt stress decreases the net photosynthesis and stomatal conductance, affecting the availability of CO_2 for carboxylation, which is a response that a virus could also cause, since a pathogenic virus requires the ability to multiply, by the interaction of its viral proteins with plant proteins and by the suppression of the host plant defense responses to viral infection (Tatineni & Dowson, 2012).

Regarding the transpiration rate, in 'Key' lime and 'Campbell' orange, higher values were found in inoculated plants then in the control, whereas, in 'Persian' lime, the average value of inoculated plants was lower than that of the control (Figure 3). The transpiration rate affects the movement of the virus through the apoplastic pathway and the horizontal transport from the phloem to the surrounding tissues (De Schepper et al., 2013).

Plants respond to any stress, whether biotic or abiotic, through various mechanisms, including a decrease of stomatal conductance to prevent the water loss by transpiration, leaf abscission to reduce transpiring leaf

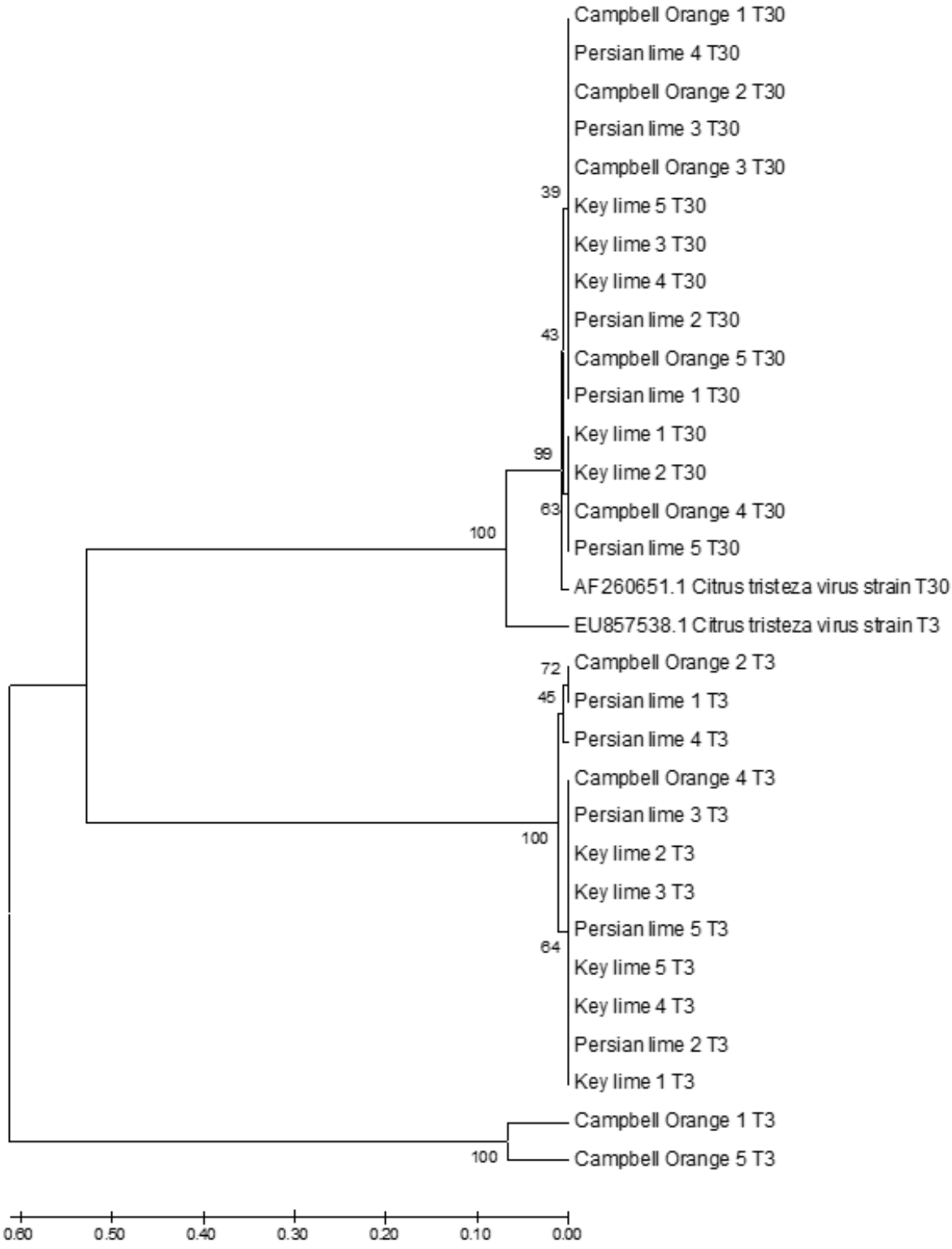

Figure 1. Phylogenetic analyses using 29 sequences from this study and the T30 and T3 reference sequences obtained from the NCBI GenBank. The maximum likelihood was based on the Jukes-Cantor (JC) model with the highest log likelihood (-427,4200) and 500 boostraps.

area, and metabolic and osmoregulation adaptations (Sperry et al., 2017). The trade-off between photosynthesis and transpiration rates influences the water use efficiency.

In citrus, stomata are generally located on the underside of leaves. When the protective cells are fully turgid, the pores open, allowing of the CO_2 absorption for photosynthesis and water loss by evapotranspiration; under severe stress conditions, they may close, restricting the CO_2 exchange and water loss (Murray et al., 2016).

The photosynthetic capacity of 'Key' lime plants infected with CTV decreased after infection, according to Perez-Clemente et al. (2015). Monitored 'Key' lime plants inoculated with CTV for 10 years showed no differences for photosynthetic and transpiration rates, in comparison with noninoculated plants (Hančević et al., 2018). In the present study, plants inoculated with T30+T3 showed no changes for photosynthetic rates.

Stomatal density (SD) varied with the species, and T1 stood out with 602.55 stomata per mm^2, whereas T3 had 578.6 stomata per mm^2 (Figure 4). Previous research indicates that the reduced stomatal density could be a response from the susceptible host to avoid further virus infection, resulting in a systemic response (Murray et al., 2016). The stomata of the control plant from all species were longer. Small stomata respond faster than large ones and, combined with high stomatal density, they show high conductance under unfavorable conditions (Reyes-López et al., 2015).

The number of stomata differed for the citrus species and for the origin of the inoculum used (Figure 5). In 'Persian' lime this difference was more evident, as there were from 374.6 stomata per mm^2 (T2) to 573 stomata per mm^2 (T1), while in 'Key' lime there were from 556.3 stomata per mm^2 (T2) to 668.8 stomata per mm^2 (T3); for 'Campbell' orange, the lowest average was 543.6 stomata per mm^2 (T3) and the highest one was 664.2 stomata per mm^2 (T2). Berdeja-Arbeu et al. (2010) reported that 'Persian' lime

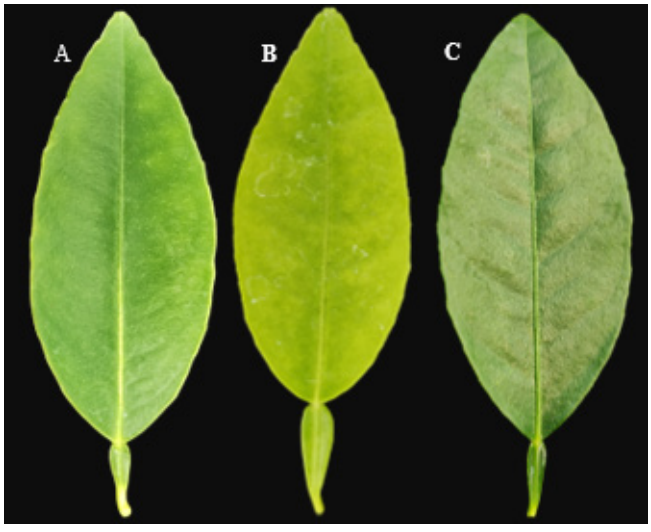

Figure 2. Symptoms in 'Key' lime (*Citrus aurantifolia*) leaves subjected to the inoculation of *Citrus tristeza virus* isolates T30+T3 genotypes: A, slight yellowing of leaf veins; B, generalized chlorosis; and C, asymptomatic leaf. Photos by Rosalba Contreras Maya.

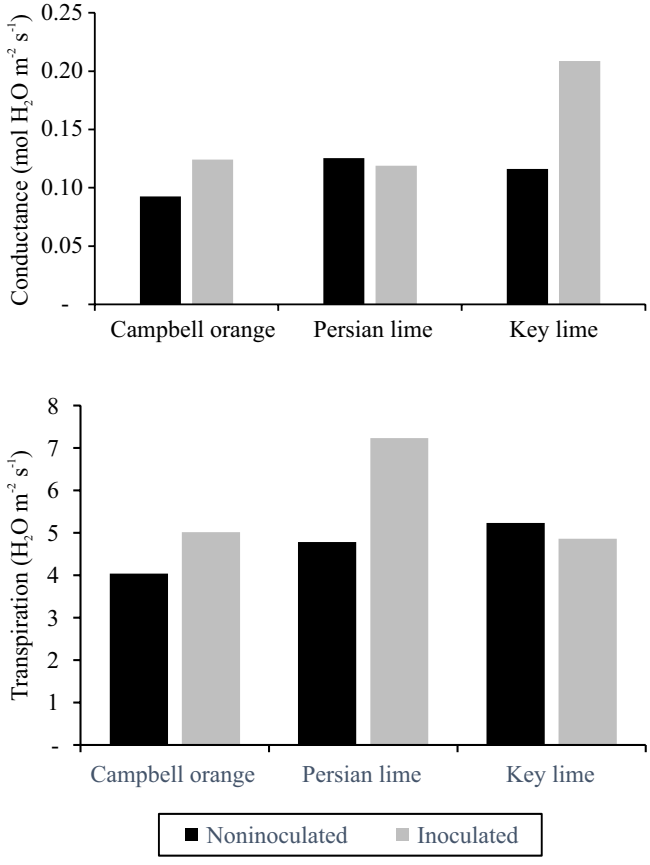

Figure 3. Stomatal conductance and transpiration rate in the citrus varieties 'Campbell' orange, 'Persian' lime, and 'Key' lime inoculated and noninoculated (T0) with *Citrus tristeza virus* isolates T30+T3 genotypes.

grafted on 'Volkameriana' lime showed the average of 328 stomata per mm^2, whereas orange leaves on 'Volkamer' lemon had the average of 106 stomata per mm^2 (Arrieta-Ramos et al., 2010).

The reduction of stomatal density and index would probably be associated with reduced transpiration and increased water use efficiency by the host (Franks et al., 2015). Whether the reduction of the stomatal index is a plant defense response or benefits the virus during infection is still unknown. Virus infection generally causes morphological and physiological alterations in infected plants (Zhao et al., 2016).

In the control treatment (T0), the three citrus species showed higher values for stomata length ('Campbell' orange, 26.1 µm; 'Persian' lime, 25.7 µm; and 'Key' lime 22.2 µm) than the plants in treatments T1, T2, and T3, which showed values lower than 24.3 µm for the three species (Figure 6). Arrieta-Ramos et al. (2010) found that orange grafted on 'Volkamerian' lemon had stomata measuring on average 23.92 × 19.99 µm (length × width); while Berdeja-Arbeu et al. (2010) mentioned that in 'Persian' lime/ 'Volkamerian' lemon, the length of stomata was 24.67 µm. In the present study, the stomata size decreased, but a possible photosynthesis rate and yield reduction need further research.

A greater average stomatal width was observed in 'Persian' lime (22 µm) and 'Campbell' orange (21 µm) from the T0 treatment; however, for 'Key' lime, T0 (18.4 µm) was lower than the plants in T1, T2, and T3 (Figure 6). It is possible to confirm, by the results obtained, that the virus affected the stomatal length in the three species, and the stomatal width in 'Persian'

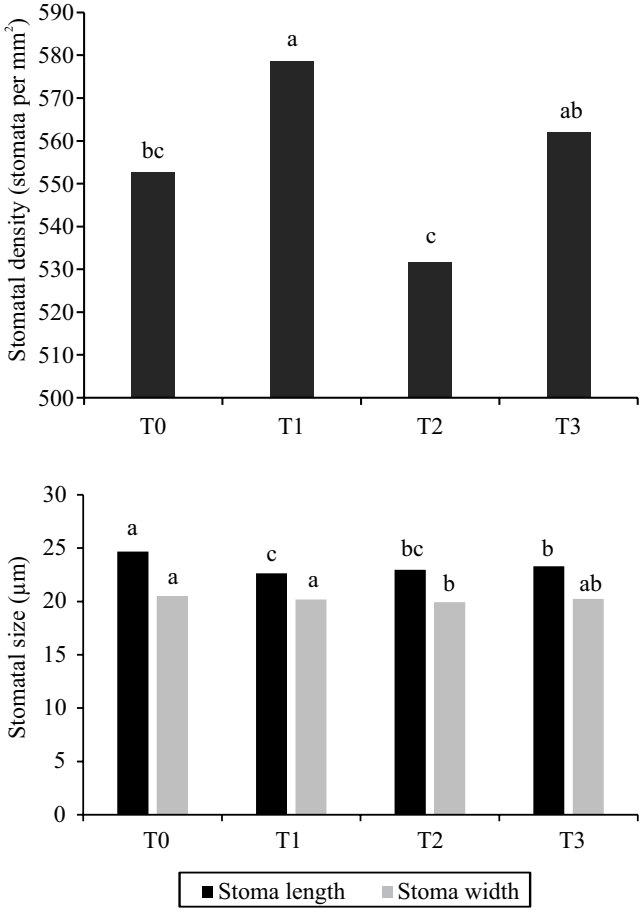

Figure 4. Stomatal density (mean number of stomata per mm^2) and stomatal size (length and width) of the citrus varieties 'Campbell' orange, 'Persian' lime, and 'Key' lime inoculated and noninoculated (T0) with *Citrus tristeza virus* isolates T30+T3 genotypes, from the inocula sources, as follows: T1, orange 1, Rancho Nuevo; T2, orange 2, Rancho Nuevo; T3, orange 3, Castillo de Teayo. Means with equal letters in the columns for each variable, do not differ by Tukey's HSD test, at 5% probability.

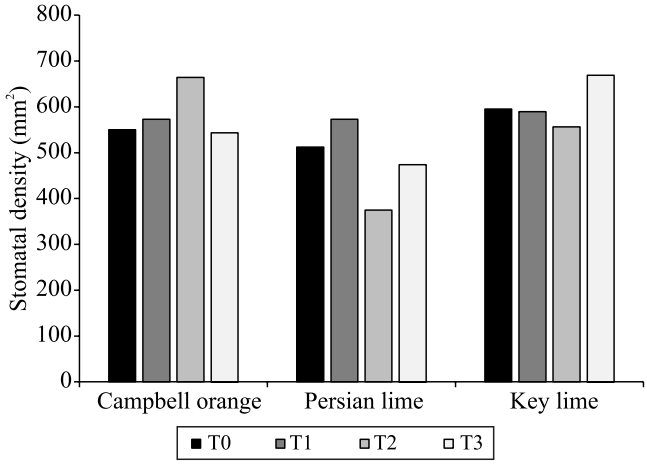

Figure 5. Mean stomatal density per mm^2 in the citrus varieties 'Campbell' orange, 'Persian' lime, and 'Key' lime inoculated and noninoculated (T0) with *Citrus tristeza virus* isolates T30+T3 genotypes, from the inocula sources, as follows: T1, orange 1, Rancho Nuevo; T2, orange 2, Rancho Nuevo; T3, orange 3, Castillo de Teayo.

lime and 'Campbell' orange. It is necessary to know if the observed changes affect the photosynthesis and yield of each species.

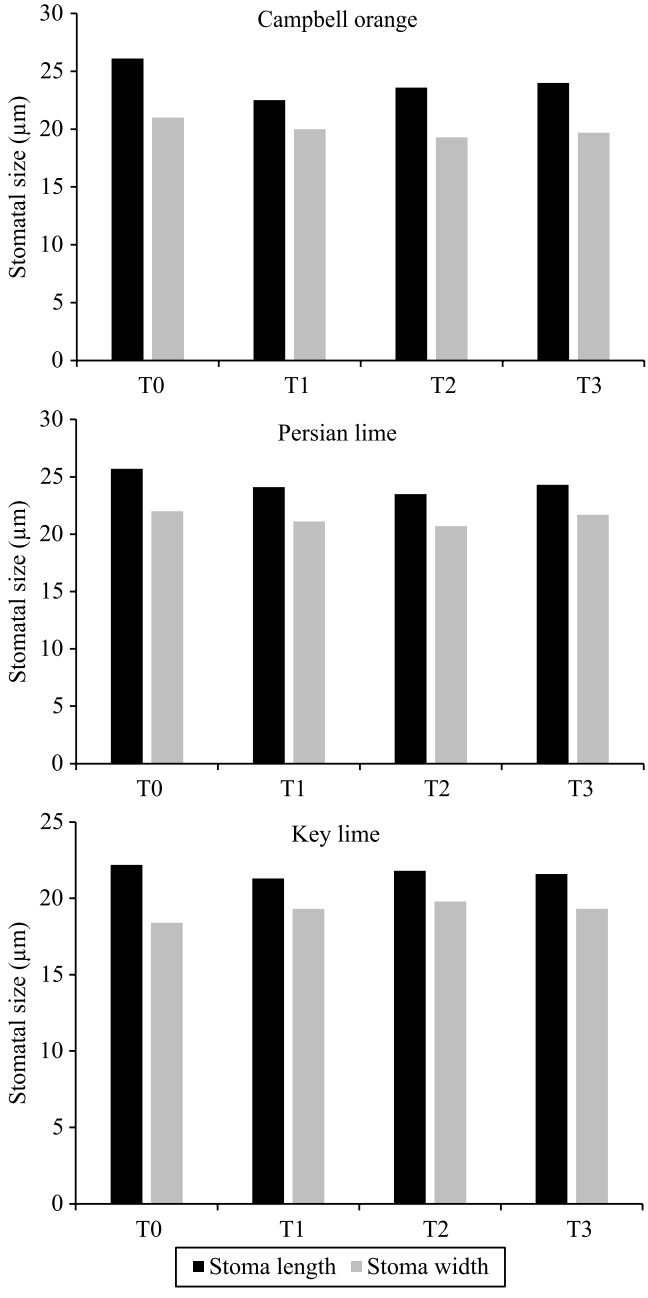

Figure 6. Mean of length and width stomatal size of the citrus varieties 'Campbell' orange, 'Persian' lime, and 'Key' lime inoculated and noninoculated (T0) with *Citrus tristeza virus* isolates T30+T3 genotypes, from the inocula sources, as follows: T1, orange 1, Rancho Nuevo; T2, orange 2, Rancho Nuevo; T3, orange 3, Castillo de Teayo.

Conclusion

The T30+T3 isolates do not affect the photosynthetic response of 'Campbell' orange and 'Persian' lime and have the potential to provide cross-protection to citrus varieties against severe isolates of *Citrus tristeza virus* (CTV) in citrus.

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