Acta Scientiarum

http://www.periodicos.uem.br/ojs/
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v46i1.63165

Transmission of isolates of *Citrus tristeza virus* by single aphids (*Toxoptera citricida*)

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ABSTRACT. *Citrus tristeza virus* (CTV) can be transmitted by several aphid species in a semi-persistent mode, with *Toxoptera citricida* being the most efficient vector. In Brazil, mild CTV isolates are used for pre-immunization of citrus trees against severe isolates. We aimed to determine the capacity and efficiency of *T. citricida* in separating the viral complex into haplotypes from three well-characterized CTV isolates (PIAC, CS1, and Forte Rolândia). Single-aphid transmission assays were conducted to determine CTV transmission efficiency. The results showed that *T. citricida* transmitted only haplotypes from the PIAC and CS1 isolates, with efficiencies of 8 and 4%, respectively. Both isolates caused mild CTV symptoms in Brazil. However, isolate Forte Rolândia, which causes severe symptoms in citrus trees, was not transmitted by *T. citricida*. The detection of CTV haplotypes from PIAC and CS1 isolates in sweet orange (*Citrus sinensis*) plants after a single aphid transmission occurred at different time points. The first haplotype observed was from PIAC 150 days after citrus was challenged by a single aphid, followed by CS1 at day 210 after transmission. In addition, differences in the single-strand conformation polymorphism patterns between the CTV isolates and CTV haplotypes were determined, suggesting that an aphid can acquire and transmit only one CTV haplotype to citrus plants during phloem feeding. The study of the mechanism of transmission by the vector can increase our knowledge of the interactions among hosts, vectors, and pathogens, which are often neglected.

Keywords: *Citrus tristeza* disease; viral transmission efficiency; *Closteroviridae*; aphid; single-strand conformation polymorphism (SSCP).

Received on April 5, 2022.
Accepted on August 6, 2022.

Introduction

Viruses are responsible for 47% of emerging diseases worldwide. Over 2,000 species of plant viruses are classified into approximately 23 families, causing diseases that affect many economically important crops and resulting in significant losses. These infections threaten agriculture worldwide (Anderson et al., 2004). Citrus species are among the most important fruit crops, with a cultivated area of 7.2 million hectares worldwide (Savita & Nagpal, 2012). Several viral diseases affect citrus, but the most important is citrus tristeza, caused by *Citrus tristeza virus* (CTV) (Lee & Keremane, 2013), which has destroyed millions of citrus trees in the last few decades worldwide (Bar-Joseph, Marcus, & Lee, 1989; Moreno, Ambrós, Albiach-Marti, Guerri, & Peña, 2008).

CTV is a member of the *Closteroviridae* family of the genus *Closterovirus*. The virus consists of a single, positive-stranded RNA genome enveloped in a helical-shaped, long, flexuous protein particle measuring approximately 11 × 2,000 nm in size (Kitajima, Silva, Oliveira, Müller, & Costa, 1964; Bar- Joseph et al., 1989; Muniz et al., 2012). Citrus is a graft-propagated perennial crop, and CTV is readily graft-transmissible. CTV comprises a complex of haplotypes (or strains) and isolates, and is spread semi-persistently by aphid vectors. Therefore, CTV populations in citrus trees are quasispecies in nature, with a complex mixture of viral genotypes subject to extensive recombination between lineages, allowing the evolution of new genotypes (strains) that have subsequently been retained within the global CTV populations on aphids and citrus trees (Harper, 2013). Citrus trees can become infected with multiple CTV strains over time (Yokomi, 2015), resulting in a viral complex. The composition of the viral complex determines the pathogenicity and transmission efficiency of the vector (Dawson et al., 2013).

Several CTV isolates have been identified and classified according to their presence or absence and the severity of symptoms that develop on citrus indicator species (Hilf, Mavrodieva, & Garnsey, 2005). Common

symptoms of CTV-infected plants include stunting, stem-pitting, slow decline, quick decline, and/or plant death (Dawson et al., 2013). The range of CTV-induced symptoms is dependent on many factors, including genetic diversity of CTV isolate, citrus cultivar, rootstock, and environmental conditions (Powell, Pelosi, Rundell, & Cohen, 2003; Moreno et al., 2008; Giampani et al., 2017).

CTV is transmitted in a semi-persistent manner by several aphid species, including *Aphis gossypii* (Glover), *Aphis spiraecola* (Patch), and *Toxoptera citricida* (Kirkaldy) (Costa & Müller, 1980; Herron, Mirkov, Da Graça, & Lee, 2006; Roistacher & Bar-Joseph, 1987). Among them, *T. citricida*, known as the brown citrus aphid, is considered the most efficient and specialized vector of CTV (Yokomi, 2015; Costa, Nunes, Zanutto, & Müller, 2010). CTV acquisition and transmission by *T. citricida* require feeding times ranging from minutes (5 to 10) to several hours (24) (Michaud, 1998; Yokomi, 2009).

In Brazil, the presence of *T. citricida* was first recorded in 1937, and subsequently, the vector and the virus spread throughout citrus-producing regions, destroying millions of trees grafted onto sour orange rootstocks (Roistacher & Bar-Joseph, 1987; Moreno et al., 2008). Studies on the tri-trophic interaction 'citrus-aphid-CTV' have demonstrated that the efficiency of transmission using a single aphid varies from 0 to 55% (Broadbent, Brlansky, & Indsto, 1996; Lin, Brlansky, & Powell, 2002; Whitfield, Falk, & Rotenberg, 2015). However, the efficiency of transmission of CTV isolates and sub-isolates under environmental conditions in southern Brazil is not known. Thus, the objectives of this study were to: i) determine the transmission efficiency of three CTV isolates (PIAC, CS1, and Forte Rolândia) previously identified in citrus groves located in Parana, Brazil, and ii) detect and identify genomic differences using single-strand conformation polymorphisms (SSCP) in isolates of CTV from CTV-infected citrus plants.

Material and methods

Citrus plants

Experiments were performed in a greenhouse at the Núcleo de Pesquisa em Biotecnologia Aplicada (NBA) in Paraná, Brazil (23°23'57.8" S, 51°57'05.3" W and altitude of approximately 500 m). Plants were maintained at 35 ± 5°C with a photoperiod of 16h. The experiments used two-year-old sweet orange (cv. Pêra) (*Citrus sinensis* (L.) Osbeck) grafted onto Rangpur lime (*Citrus limonia* Osbeck) rootstock. Plants were maintained in 4-liter polypropylene pots, with dimensions of 15 × 15 × 35 cm (Multipote-JKS Industrial, SP, Brazil) and grown on a commercial substrate (Basaplant Citrus - Base Agro – SP, Brazil). Plants were irrigated daily.

Virus isolates

In this study, two different sources of isolates were used, termed 'CTV isolates' and 'CTV haplotypes'. These have been used in single-aphid transmission studies. Three CTV isolates were used in the study, two of which have been described as mild-protective isolates, PIAC and CS1 (Carraro, Nunes, Corazza-Nunes, Machado, & Stach-Machado, 2003; Zanutto, Corazza, Nunes, & Müller, 2013), and one isolate that caused severe symptoms, Forte Rolândia (Zanutto et al., 2013). Isolate PIAC was provided by the Center of Citriculture Sylvio Moreira (Cordeirópolis, São Paulo State, Brazil) in 2015. Isolate CS1 was obtained from a commercial citrus nursery (Viveiro de Mudas Pratinha) located in the northwest of Paraná State, Brazil (Paranavaí county, 23°03'08.1" S 52°26'28.2" W) where the isolate is kept in parent plants. Forte Rolândia was obtained from commercial citrus orchards in the northern region of Paraná State, Brazil (Rolândia county, 23°20'03.5" S, 51°27'08.8" W).

Insect colonies

Brown citrus aphids were collected from experimental citrus orchards located in the *Núcleo de Pesquisa em Biotecnologia Aplicada* (NBA, Brazil) in the summer of 2016. Groups of 100 aphids were transferred and reared into four 2-year-old healthy Pêra sweet orange plants, which were kept in insect-proof cages in a greenhouse maintained at 35 ± 5°C (environmental conditions) with a photoperiod of 16h until needed for experiments.

Virus acquisition, transmission, and colonization assays

Acquisition and transmission assays were conducted using a previously published protocol (Brlansky, Damsteegt, Howd, & Roy, 2003; Korkmaz et al., 2022). Briefly, more than 40 adult insects were collected from

the insect source colonies and transferred to healthy 2-year-old Pêra sweet orange plants for 72h to cleanse viral particles present in the aphid's alimentary canal. Once the cleansing period was complete, acquisition assays were performed by transferring new groups of 40 uninfected adult aphids to a CTV-infected Pêra sweet orange seedling (inoculation occurred 5 months before assays were initiated and confirmed by PCR analysis) with either one of the three CTV-isolates PIAC, CS1, or Forte Rolândia. Aphids were placed on the seedlings for an acquisition access period of 48h. To perform the CTV transmission assays, we followed the protocol described by Herron et al. (2006) and Hernández-Rodríguez et al. (2019), in which a single aphid was transferred from the infected plant with one of the CTV-isolates to a healthy seedling of the variety Pêra for an inoculation access period of 48 hours. There were 25 biological replicates per CTV isolate. After the inoculation access period, aphids were killed using an insecticide spray (Dimethoate, FMC Corporation, Brazil). CTV-inoculated plants were then placed and maintained in a greenhouse at 35 ± 5°C with a photoperiod of 16h. CTV colonization of the Pêra plants was monitored by sampling the central vein of three leaves collected at different canopy heights (upper, middle, and lower) and analyzing them together. Samples were collected every 30 days from 30 to 270 days post inoculation (nine times). The transmission efficiency was determined by dividing the number of CTV-positive plants by the total number of plants challenged with each CTV isolate.

RNA extraction and cDNA synthesis

Plant tissues (central veins) were collected and pooled from the upper, middle, and lower canopy leaves, flash-frozen in liquid nitrogen, and immediately homogenized mechanically with a mortar and pestle. After homogenization, 200 mg of plant material (frozen powder) was used for RNA extraction, which was performed using 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was quantified using a spectrophotometer (Mod. UV5; Mettler Toledo, USA) at 260 nm, and RNA quality was assessed on a 1% agarose gel stained with ethidium bromide.

Reverse transcription to generate complementary DNA (cDNA) was performed using 300 ng of total RNA (template) in a final reaction volume of 25 µL. The cDNA reaction synthesis included: 5X first-strand buffer (Invitrogen), 2.5 mM of each deoxynucleotide (dNTPs), 10 mM dithiothreitol (DTT), 20 units of RNase inhibitor (Invitrogen), 1 µg of random primers, and 100 units of M-MLV reverse transcriptase (Invitrogen). The mixture was incubated at 37°C for 2h, and the cDNA samples were stored at -20°C until further analysis could be conducted.

CTV-detection assay

To detect CTV, samples were subjected to polymerase chain reaction (PCR) to detect viral gene *capsid protein p25* (GCP). The PCR reaction contained the following reagents: 100 ng of cDNA template, 10X DNA polymerase buffer (Invitrogen), 2.5 mM of $MgCl_2$, 2.5 mM of each dNTP, 100 ng of each specific primer CN-119 and CN-120 (Cevik et al., 1996), and 1 unit of Taq DNA polymerase (Invitrogen) in a total volume of 25 µL. The PCR conditions consisted of a denaturation step at 95°C for 2 min., followed by 35 cycles of 95°C for 1 min. 55°C for 1 min., and 72°C for 2 min., and a final extension at 72°C for 5 min. The resulting PCR amplicons were analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide. Images of each gel were captured using an ultraviolet gel documentation system (UVP Bioimaging Systems, Analytik Jena US LLC, Upland, CA, USA).

Single Stranded Conformational Polymorphism (SSCP) analysis

Once PCR detection confirmed the presence of CTV, SSCP analyses were performed to evaluate the differences in the electrophoretic patterns of each PCR amplicon obtained using the methodology described by Souza, Müller, Targon, and Machado (2000) and Corazza-Nunes et al. (2006). Briefly, SSCP analysis was performed using 10 µL of each PCR amplicon mixed with an equal volume of denaturing buffer (95% formamide, 2 mM EDTA, and 0.05% bromophenol blue). Samples were incubated at 95°C for 10 min. (denaturation) in a thermal block and placed immediately on ice. DNA samples were loaded on 8% polyacrylamide non-denaturing gels, and electrophoresis was performed in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.5) at 200 volts for 16h at 25°C. After electrophoresis, gels were stained with a solution of silver nitrate according to the procedure described by Beidler, Hilliard, and Rill (1982). Images of each gel

were documented, and the SSCP patterns were analyzed in relation to the number of bands observed in each lane to determine the CTV isolate or haplotype identity.

Results

Detection of CTV isolates on citrus plants

The presence of CTV in citrus plants used as a source of different isolates (PIAC, CS1, and Forte Rolândia) was analyzed by PCR amplification. Our results confirmed the presence of the gene *capsid protein p25*, confirming that all plant materials used for single-aphid transmission assays were CTV-infected (Figure 1).

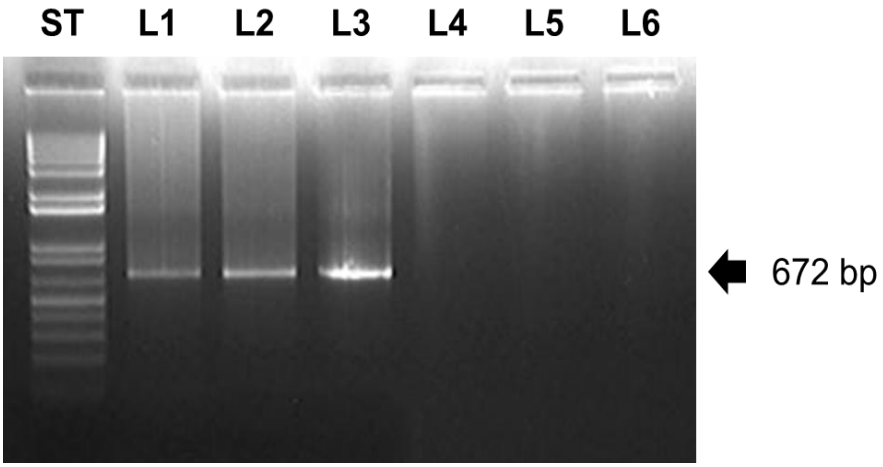

Figure 1. PCR amplification of the *capsid protein p25* gene from plants infected with CTV isolates PIAC, CS1, and Forte Rolândia used as a source of each isolate. The image shows the DNA amplicon of the expected size, 672 bp. ST is the 1 Kb Plus DNA Ladder (Invitrogen – USA), lanes 1 to 3 correspond to the aforementioned CTV isolates, and lanes 4 to 6 are the negative controls.

Colonization time of CTV isolates

The colonization (or incubation) time for each CTV isolate (PIAC, CS1, and Forte Rolândia) was analyzed over a 270-day period. PCR results showed that the *capsid protein p25* was successfully identified in two out of three CTV isolates, indicating differences in colonization time among the isolates (Figure 2). 'PIAC' was the first CTV isolate detected 150 days after aphid transmission assay. This was followed by the isolate CS1, which was detected on day 210. The more severe isolate, Forte Rolândia, was not detected at any time post-inoculation (maximum sample time, 270 days). These results were also verified by visual inspection of the plant symptoms associated with CTV infection at the end of the experiment.

SSCP polymorphism after CTV transmission

The pattern of each CTV-positive amplicon sample was analyzed using SSCP (Figure 3). The SSCP patterns for the CS1, PIAC, and Forte Rolândia CTV isolates are presented in Figure 3A. In detail, the isolate 'CS1' had a pattern of 5 polymorphic bands, comprising 3 strong and 2 faint bands, while the 'PIAC' isolate had 3 distinctive bands, including 1 strong and 2 faint bands, while 'Forte Rolândia' isolate had a total of 5 polymorphic bands, consisting of 4 strong and 1 faint bands. The CTV haplotype amplicon fingerprints of the experimental plants obtained using a single-aphid transmission assay are presented in Figure 3B. The CS1 haplotype had only three 3 strong bands, whereas the PIAC haplotype had 1 strong and 1 faint lower band (bands are indicated with numbers).

Efficiency of single-aphid transmission of CTV

The transmission assays were performed using a single aphid per plant (Table 1). Our results showed that one plant was positive after transmission from a single aphid carrying the 'CS1' CTV haplotype with a rate of transmission of 4%, and two plants were positive after transmission from single aphids carrying the 'PIAC' CTV haplotype, with a rate of transmission of 8%. No viral transmission was observed in plants inoculated with Forte Rolândia isolate during this experiment.

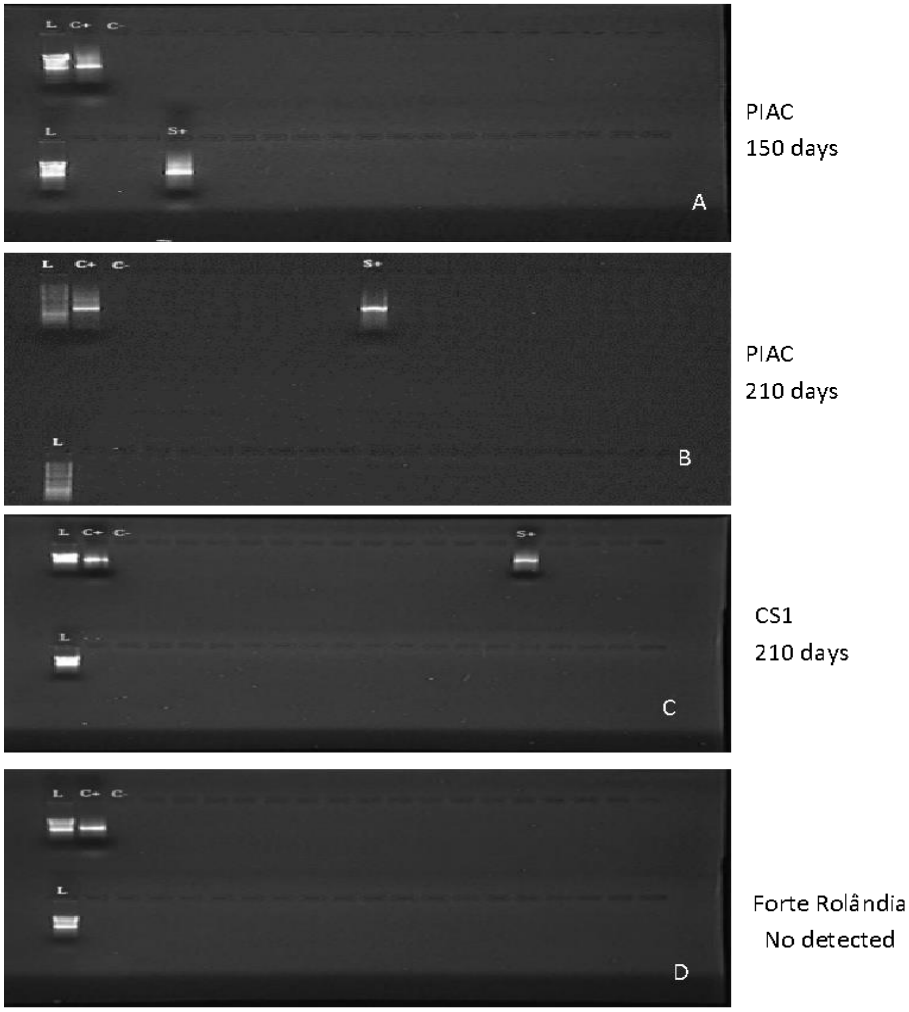

Figure 2. Detection of CTV isolates (PIAC, CS1, Forte Rolândia) after single-aphid transmission in each one of the 25 individual citrus plants per viral isolate over time. Representative images are of CTV detection by PCR at different time points after transmission. The first detection was at day 150 in trees with aphids transmitting the isolate PIAC (1 positive sample - A) and at day 210 for trees with aphids transmitting the isolate PIAC (B) and CS1 (C) (1 positive sample). No CTV was detected in trees with aphids transmitting the isolate Forte Rolândia (D). (L) 1 Kb Plus DNA Ladder (Invitrogen – USA), (C+) corresponds to positive control, (C-) is negative control, and (S+) is a positive sample.

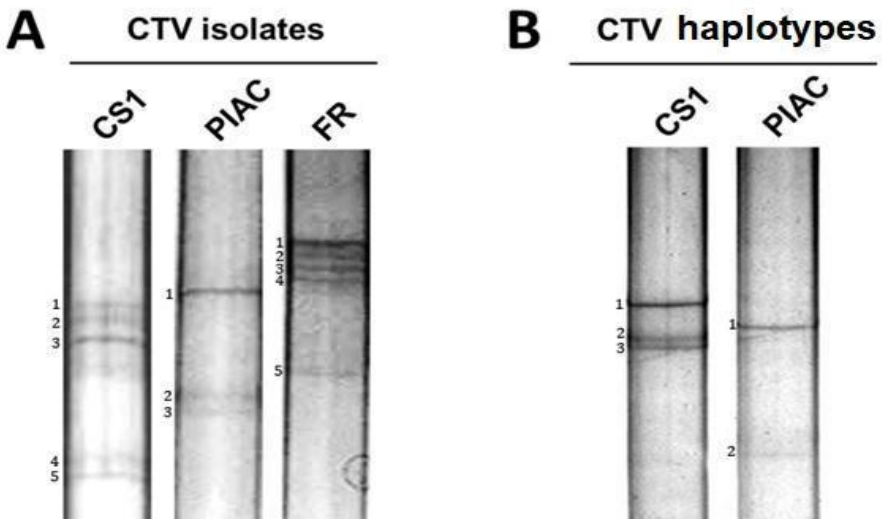

Figure 3. Single-Stranded Conformational Polymorphism patterns of CTV isolates and CTV haplotypes. (A) SSCP patterns identified in citrus plants infected with the three CTV isolates, CS1, PIAC, and Forte Rolândia (FR), used as source plants. (B) SSCP patterns of CTV haplotypes identified in citrus plants infected by single-aphid transmission with only one isolate or haplotype. Only haplotypes of CS1 and PIAC were identified.

Table 1. Transmission rate of CTV isolates (CS1, PIAC, and Forte Rolândia) using single-aphid (*Toxoptera citricida*) transmission.

CTV isolate	Positive / inoculated plants
CS1	1/25
PIAC	2/25
Forte Rolândia	0/25

Discussion

The present study aimed to assess the transmission rate of three CTV isolates (PIAC, CS1, and Forte Rolândia) using single-aphid (*T. citricida*) transmission under partially controlled greenhouse conditions. Among the three isolates, PIAC and CS1 provided protection against aggressive isolates of CTV in Pêra sweet orange (Müller, 1980; Zanutto et al., 2013). The CTV isolate Forte Rolândia has been identified as an aggressive viral complex that induces severe citrus tristeza disease symptoms and causes significant economic damage to commercial citrus orchards in the northern regions of Paraná State (Carraro et al., 2003; Zanutto et al., 2013). CTV-isolates consist of a population of haplotypes that are highly divergent (Ruiz-Ruiz, Moreno, Guerri, & Ambrós, 2007; Leonel et al., 2015). The composition of genetic types in a CTV-infected plant may vary because of the high rate of mutation (Corazza-Nunes, Zanutto, Zanineli-Ré, Müller, & Nunes, 2012) or repeated acquisitions as a result of multiple transmission events with different CTV haplotypes (Ayllón et al., 2006). In this acquisition-transmission study, we used three CTV isolates (PIAC, CS1, and Forte Rolândia) to infect citrus plants using single aphids, and we determined that insects can acquire and transmit single CTV haplotypes that appeared to have different transmission and/or colonization times. The SSCP patterns indicated variation in CTV-infected samples from the parent type.

Our results indicate differences in the efficiency of transmission of *T. citricida* between two mild isolates of CTV (PIAC and CS1) and a severe isolate (Forte Rolândia). The CTV isolates PIAC and CS1 showed an efficiency of transmission of 8 and 4%, respectively. Forte Rolândia was not detected in any of the recipient plants during the experiment. These results corroborate those of a previous study, in which the efficiency of CTV transmission ranged from 0 to 3.6%. However, a higher efficiency of transmission was determined for severe CTV isolates under greenhouse conditions in Florida, United States (Lin et al., 2002). The low rate of CTV transmission that we observed for the mild isolates PIAC and CS1 might be a consequence of the high temperatures (~34°C) in Maringa, Brazil (the studies were conducted in a greenhouse at 35 ± 5°C). Studies have evaluated the effect of temperature on transmission; for instance, in Madame vinous sweet orange exposed to different temperatures, CTV transmission by the melon aphid (*Aphis gossypii*) was 12.2 to 60.8% (Bar-Joseph & Loebenstein, 1973). Similarly, Korkmaz et al. (2022) reported that *A. gossypii* could not transmit nine isolates in repeated experiments. While some isolates were transmitted to only one seedling, other isolates were transmitted to multiple seedlings, and the average transmission efficiency was 4.64%.

Our study utilized single aphids for transmission, and other studies have demonstrated that using more than one *T. citricida* aphid can result in transmission rates for CTV ranging from 0 to 59% (Broadbent, 1996; Lin et al., 2002; Herron et al., 2006).

In addition to environmental conditions, other reasons for the low rates of CTV transmission may involve capsid proteins. The transmission process of some non-circulating virus species, of which CTV is an example, may require that other factors encoded by the virus for effective transmission, called "helper" proteins, are present for successful survival and transmission of the vector (Pirone & Blanc, 1996; Herron et al., 2006). This association has been described for *Cauliflower mosaic virus* (CaMV), which is transmitted by aphids in a non-circulatory manner. CaMV uses a 'helper' strategy during the transmission process. In this case, two proteins with a plausible role had been identified as 'helper proteins'; these proteins are non-structural viral proteins (P2 and P3) that assist in the adhesion and retention of viral particles in the cuticle of the aphid stylets, promoting effective transmission (Leh et al., 2001; Palacios et al., 2002). In the case of CTV, several genes have been suggested as 'helper' gene candidates (Herron et al., 2006), and the proteins they encode assist with the adhesion and retention of CTV particles in the aphid, *T. citricida* (Bar-Joseph, Gumpf, Dodds, Rosner, & Ginzberg, 1985; Karasev et al., 1995; Flores et al., 2013). Other factors can influence plant interactions. Plants possess defense mechanisms against viral single-stranded RNAs (ssRNAs). The defense response occurs when plants synthesize small interfering RNAs (siRNAs) and microRNAs (miRNAs) that align with viral RNAs (Cheng et al., 2015). However, this process can be nullified by viruses that block the expression of RNA silencing suppressors (RSSs) (Mette, Matzke, & Matzke, 2001; Groenenboom & Hogeweg, 2012). Indeed, in

the CTV genome, the gene products p20, p23, and p25, have been identified as proteins involved in the suppression of RSSs (Lu et al., 2004), therefore it seems that the low rate of transmission observed in this study was not caused by plant defense mechanisms.

The differences in colonization times between the PIAC and CS1 isolates, where the detection times were 150 and 210 days, respectively, could be due to many factors. The main causes include the amount of host tissue used for CTV detection, the heterogeneity of the CTV distribution in the infected plant, and differences in specificity of the primers used for CTV identification, as was previously reported (Nolasco, Fonseca, & Silva, 2008). Modifications in the SSCP patterns between CTV isolates and CTV haplotypes were identified (Figure 3), and the differences in the SSCP patterns for CTV haplotypes were possibly caused by the use of single aphids to perform the transmission. It is accepted that one aphid can acquire only one haplotype at a time, by a process known as segregation (Broadbent et al., 1996; Tsai, Liu, Wang, & Lee, 2000; Brlansky et al., 2003; Herron et al., 2006; Lin et al., 2002). This was observed in a field study, in which aphids (*T. citricida*) fed on CTV-infected plants transmitted different haplotypes to citrus test plants, with the sources being determined by different SSCP patterns (Nolasco et al., 2008), as was observed in our study.

Conclusion

This is the first study conducted in Brazil on single-vector transmission of CTV using two different isolates. Transmission assays showed that the mild CTV isolates were transmitted with low efficiency, while the severe CTV isolate was undetected. In addition, the SSCP patterns varied between the mild CTV isolates and their respective haplotypes obtained after single-aphid transmission. Future studies to evaluate in vitro acquisition and detection in aphid stylets before and after transmission of CTV isolates will help us to understand the mechanisms that occur in aphid-virus interactions, with the aim of reducing the economic impact associated with citrus Tristeza disease.

Acknowledgments

This research received financial support from the National Council for the Improvement of Higher Education (CAPES) for the scholarship granted to the first and third authors and from the National Council for Scientific and Technological Development (CNPq) for the productivity scholarship granted to the sixth author (Process 302809/2022 -8 -CNPq).

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