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ORIGINAL ARTICLE

The effect of drought conditions on sweet orange (*Citrus sinensis*) plants infected with citrus tristeza virus (CTV)

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Abstract
Brazil is one of the world's largest producers of citrus. However, production is challenged due to biotic and abiotic factors limiting crop health. The aim of this work was to evaluate the influence of water deficit in sweet orange (*Citrus sinensis*) cv. Pera Bianchi inoculated with citrus tristeza virus (CTV). Two isolates of CTV were used, one causing severe symptoms (Forte Rolândia) and the second causing mild symptoms (Pêra IAC), grafted on Rangpur lime (*Citrus limonia*) and Swingle citrumelo [*Citrus paradisi* x *Poncirus trifoliata*], and the indicator sweet orange Pêra Bianchi, free of virus, and healthy controls containing only the indicator budwood. The water regime for the plants was field capacity or 50% field capacity. After five months of controlled irrigation, biochemical variables were measured (protein, proline content and catalase activity), and real-time RT-PCR amplification of the virus was performed for detection and quantification of viral titer. Differences were observed in the total protein content and proline, with greater accumulation in plants maintained under water deficit. There was no effect of drought on the population of viral isolates, but the plants held at field capacity and inoculated with the severe isolate had a higher viral titer.

Keywords Drought · Plants stress · Proline · Viral replication

Introduction

Citrus plants are exposed to abiotic and biotic factors that can influence the productivity of the crop (Li et al. 2006; Syvertsen and Hanlin 2008). Among abiotic stresses, drought is particularly serious, because it affects the growth and development of the plants directly, as well as fruit production and quality (Salem-Fnayou et al. 2016). Compared with other plants of the same photosynthetic group (C3), the water use efficiency of citrus plants is low and studies have shown that fruit loses water to the leaves during periods of water deficiency (Davies and Albrigo 1994). Depending on the water deficit conditions, the leaves curl, reducing the area of transpiration; mature fruit dehydrate by establishing an inverse flow (from the leaves to the fruit), and a significant quality change occurs, with an increase in solids. The leaves and fruit can fall and in extreme cases the plant may die (Zanini et al. 1998).

Of all abiotic environmental factors, availability of water is probably the most limiting abiotic stress for crop quality and productivity in citrus. In response to drought, the citrus plant can activate various protection mechanisms, including osmotic adjustment, which is one of the main physiological mechanisms related to plant defense against water shortage. Osmotic adjustment is characterized by the accumulation of non-enzymatic compounds in the plant cells, which are known as compatible solutes (Laborem et al. 1991; Marijuan and Bosch 2013). Among them, proline is a well characterized molecule that acts on osmotic adjustment, subcellular structures stabilization and the elimination of free radicals, besides participating in nitrogen and carbon stock, that could be used after the period of stress (Taylor 1996; Leite et al. 2000).

Furthermore, under stress conditions (whether abiotic or biotic), an oxidative burst can occur in the stressed plant very rapidly, consisting of the accumulation of reactive oxygen species (ROS), mainly superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) ions (Hegedus et al.

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2001; Hu et al. 2009). To protect themselves from this burst of oxidative radicals, organelles including chloroplasts, mitochondria and peroxisomes use an antioxidant defense system, which includes the production of enzymes that will eliminate ROS, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Nascimento and Barrigossi 2014).

Among biotic factors that cause plant stress, diseases associated with plant pathogens are an important and recurrent problem that limits citrus productivity in Brazil. Studies suggest that an increase in the reactive oxygen species concentration, mainly H_2O_2, is related to the decrease of the plant pathogen population (Kumar et al. 2011a, 2011b). In response to the pathogen attack, the plant develops a defense system which encompasses several reactions associated with programmed cell death, hypersensitivity response and the action of oxidative metabolism-related enzymes (Kumar et al. 2011a). The enzymatic defense system consists of components responsible for removing, neutralizing or eliminating free radicals within the cells, and within this group is the CAT enzyme (Scandalios 1993; Wojtaszek 1997; Kumar et al. 2011b). This enzyme plays an important role in defense mechanisms, mostly those related to hypersensitivity and programmed cell death (Van Breusegen et al. 2001; Vellosillo et al. 2010).

Citrus tristeza, caused by citrus tristeza virus (CTV) is considered one of the most important diseases affecting citriculture worldwide (Moreno et al. 2008). *Citrus tristeza virus* is a species in the genus *Closterovirus* of the family *Closteroviridae*. The virus particles are flexuous and filamentous, approximately 10–12 nm in diameter and 2000 nm in length, consisting of a single-stranded RNA molecule with positive polarity. The genetic material is encapsulated in a capsid formed by p25 and p27 (Kitajima et al. 1964; Bar-Joseph et al. 1989; Karasev et al. 1998).

The CTV genome has 19,226 to 19,296 nucleotides, with 12 open reading frames (ORFs) encoding at least 19 proteins, and two untranslated regions (UTRs) of approximately 107 and 273 nucleotides at the 5′- and 3′-end, respectively, which are regions necessary for the replication of the genomic RNA (Karasev et al. 1998; Satyanarayana et al. 1999). The 3'-UTR is the most conserved sequence of CTV genotypes, usually with >95% similarity, while the 5'-UTRs have little sequence similarity (López et al. 1998).

CTV can be detected in plants by several methods, includ ing symptomatology (Bennet & Costa 1949; Müller et al. 2005), electron microscopy (Biswas 2008), serology (Cambra et al. 1991; Carraro et al. 2003) and molecular methods (Rubio et al. 2001; Ruiz-Ruiz et al. 2006). Among the molecular methods, real-time Reverse-Trancription Polymerase Chain Reaction (RT-PCR) amplification has been widely used, mainly due to its speed, specificity, sensitivity, and ability to provide quantification combined with the possibility of automation of the analyzes (Ruiz-Ruiz et al. 2007).

Symptoms of CTV vary according to the scion-rootstock combination, as well as the virulence of the isolate and local climatic conditions (Moreno et al. 2008). Damage caused by virus isolates is associated with stem pitting symptoms (tree, trunk and branch depressions), atrophy of the plant, leaves of small size presenting chlorosis and formation of small fruits of defective conformation. In addition, the virus can also cause yellowing in plants that are very susceptible (Souza and Müller 2006).

In the host plant, CTV is limited to phloem cells (Bar-Joseph et al. 1979) and can be easily transmitted by tissue union, such as budwood grafting (Bennett and Costa 1949). In nature, the virus is transmitted semi-persistently by different species of aphids (Moreno et al. 2008). In Brazil, *Toxoptera citricida* Kirkaldy is considered to be the most efficient vector in its transmission (Meneghini 1946; Bennett and Costa 1949; Müller et al. 2005).

This disease can be controlled by pre-immunization with mild CTV isolates, which protect the plant against infection by severe isolates (Müller and Costa 1977). This technique was first used in the 1960s, and quickly became the most common control measure for citrus tristeza (Costa and Müller 1980).

The choice of scion variety, rootstock and seedlings to be planted is a decisive factor in planting trees in an orchard that will be tolerant or prevent colonization by the virus (Silva and Souza 2002; Bastos et al. 2014). According to Corazza et al. (2012), if rootstocks in the field are alraedy colonized with severe CTV isolates before being grafted and inoculated with mild isolates, the severe isolates will predominate in the area in question. Rangpur lime (*Citrus limonia* Osbeck) is the most commonly used rootstock in citriculture in Brazil due to its tolerance to CTV and its adaptation to water deficiency. Rangpur lime also has good agronomic characteristics with scion varieties, supporting high yield and good fruit quality. Also notably resistant to CTV is the rootstock Swingle citrumelo [*Citrus paradisi* x *Poncirus trifoliata* (L.) Raf.]: it supports good growth of scion material and offers resistance to the main diseases that affect citrus production. However, Swingle citrumelo is considered intolerant to drought under field conditions (Stenzel et al. 2005; Pompeu-Junior 2005).

As perennial plants, citrus plants are exposed to adverse environmental conditions such as low humidity in the soil and in the atmosphere during cultivation (Campos et al. 2011). Knowing that water stress favors the development of plant pathogens (Boyer 1995; Machado et al. 2007; Freitas-Astuá et al. 2007; Gonçalves et al. 2014), the aim of this study was to evaluate the effect of water deficit on trees of sweet orange Pera Bianchi [*Citrus sinensis* (L.) Osbeck] inoculated with CTV.

Material and methods

Viral isolates and plant material

Two CTV isolates were used for inoculations: Forte Rolândia, collected at a commercial orchard in the city of Rolândia, Paraná, and Pêra IAC, from Centro de Citricultura Sylvio Moreira (CCSM/APTA), state of São Paulo. Detection and characterization of the isolates was previously performed by means of RT-PCR and RFLP, respectively (data not shown). Forte Rolândia and Pêra IAC represent severe and mild types of CTV, respectively. As a control, virus-free plants and plants grafted only with the indicator budwood were used.

The isolates were inoculated by double-grafting (Ribeiro et al. 2005), using rootstocks of Rangpur lime and Swingle citrumelo. The Pêra Bianchi virus-free indicator was grafted just above the site of inoculation of the rootstocks. The plants were kept in a greenhouse for the development of the indicator budwood for six months, and were irrigated daily without any restriction of water. During this period no agrochemicals or fertilizers were applied. Subsequently, they were taken to walk-in growth chambers (Percival Scientific, AR-1010 model), where the experiment was conducted. The environmental conditions were 20 °C with a 12 h photoperiod. Plants were kept in clay soil in 7-L plastic pots. Nine months after budding and grafting was completed, the plants were subject to water stress by controlling the amount of water applied by irrigation. Plants were weighed daily in order to keep them at field capacity (and therefore not stressed) or at 50% of field capacity (stressed). The field capacity established was 1.2 L of water, and 50% of the field capacity was 600 mL of water. Controlled irrigation began in February 2016 and ended in August of the same year. To maintain established water regimes, plants were weighted and irrigated daily. To obtain the value of water to be added in each pot, the plants were weighed and then the following calculation was performed: Total weight - Dry weight (pot weight + plant weight + soil weight) resulting in amount of water present in the pot. From this result, the amount of water required to reach 1.250 L or 0.650 L of water was added. The extra 50 mL of water was added so that the plant did not stay out of the established water regimes if it happened to lose more water from one day to the next. The experiment was a complete randomized block design with three replications. After five months of controlled irrigation maintaining field capacity or 50% field capacity, the measurement of the water potential of the plants was done using a Scholander type pressure pump, according to Turner (1981), to verify if the plants were stressed by drought, or whether they were free of water stress (data not shown). At this moment, stressed plants presented characteristic symptoms of water deficiency, such as reduction of development and leaf curling. Subsequent to these measurements, biochemical and molecular analysis were performed.

Biochemical analysis

Total protein, catalase and proline were quantified. Three months after initiating the controlled irrigation, four leaves were collected. For this, the first two leaves of the apex were discarded, collecting the following four leaves, which were immediately frozen in liquid nitrogen and placed in an ultrafreezer (−80 °C).

Enzymatic extracts were obtained following Silva et al. (2010). First, 1 g of plant material from each sample was ground in 10 mL of 50 mM potassium phosphate buffer pH 7.0 containing 1% polyvinylpyrrolidone 40 (*w*/*v*). Then, the material was transferred to a 15 mL Falcon tube and centrifuged for 10 min at 7500 rpm at 4 °C. After centrifugation, the supernatant from each sample was transferred to a 2 mL microtube which was stored at −80 °C until further use.

Total protein quantification was performed using the Bradford method (Bradford 1976) using three replicates for each sample. For this, 570 μL of distilled water, 30 μL of the extract and 2.4 mL of the Bradford reagent were added to 15 mL test tubes. Absorbance readings of the resulting solution were performed using an Evolution 300 UV-VIS spectrophotometer (Thermo) at 595 nm. The absorbances obtained were compared with the standard curve (stock solution of bovine albumin at 0–15 μg/μL) and the results expressed as mg of total protein/g of fresh leaf.

Quantification of proline was performed according to Bates et al. (1973), with some modifications as described by Carillo and Gibon (2011). In non-stressed plants, 500 μL of the enzyme extract was diluted in 5 mL of 3% sulfosalicylic acid. With drought stressed plants, 250 μL of the enzyme extract was diluted in 10 mL of 3% sulfosalicylic acid. Subsequently, 2 mL of the sample, 2 mL of ninhydrin acid solution (ninhydrin, acetic acid, 6 M phosphoric acid) and 2 mL of acetic acid were added to a 15 mL test tube with a screw cap. The samples were vortexed and incubated in a water bath at 100 °C for one hour, and immediately cooled in an ice bath to terminate the reaction. The readings were taken using an Evolution 300 UV-VIS spectrophotometer at 520 nm. The absorbances obtained were compared to the standard curve (100 mg of proline diluted in 1000 mL of distilled water, ranging from 0 to 80 μg/mL) and the data were expressed in μmol of proline/g of fresh matter.

Quantification of catalase was performed according to Aebi (1974) and Peixoto et al. (1999), with modifications. Two replicates were run for each sample. First, 150 μL of the enzyme extract were added to 2.85 mL of 50 mM phosphate buffer pH 7.0 with 0.1 mM EDTA, which was used to calibrate the spectrophotometer. Then, 150 μL of the enzyme extract was added to 2.85 mL of phosphate buffer containing 15 mM H_2O_2. Spectrophotometric readings were performed at 240 nm with eight cycles of 30 s each in Kinect mode using an Evolution 300 UV-VIS spectrophotometer. A molar extinction coefficient of H_2O_2 of 36.0 M/cm was used to calculate the activity of the enzyme, which was expressed in mmol H_2O_2/min/mg of protein.

Molecular analyses

After subjecting the plants to drought stress for five months, samples of the third and fourth leaves were collected to extract the total RNA from the plants, using the Trizol reagent (Invitrogen) following the manufacturer's instructions. Prior to cDNA synthesis, the samples were treated with DNase Amplification Grade I (Invitrogen) following the manufacturer's instructions, and immediately stored at −80 °C.

Reverse transcription (RT) was performed by subjecting the samples to the ThermoScript enzyme reaction (Invitrogen) to generate the cDNA. First, quantification of the samples was perfomed in a Nanodrop (Thermo) to obtain an initial RNA concentration of 1000 ng/μL. Then, 1 μL of oligo-dT_{20} and 2 μL of 10 mM dNTP mix were added to a 0.2 mL microtube, and the concentration adjusted such that the overall concentration of RNA was maintained at 1000 ng/μL, resulting in a final volume of 12 μL. The samples were incubated in a thermocycler for 5 min at 65 °C, and immediately placed on ice. Then, 4 μL of 5X enzyme buffer, 1 μL of 0.1 M DTT, 1 μL of RNase OUT and 1 μL of ThermoScript DNA polymerase were added to the tubes. The samples were incubated at 50 °C for 60 min, followed by incubation at 85 °C for 5 min. Thereafter, 1 μL of RNase H was added and the tubes were incubated at 37 °C for 20 min. The samples were stored at −80 °C.

To perform the amplification of the 3'-UTR region of CTV, qRT-PCR was performed. The samples were initially diluted to a concentration of 10 ng/μL of cDNA. The reaction contained 5 μL of Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen), 0.25 μL of the 3'UTR1/3'UTR2 oligonucleotide primer pair (Bertolini et al. 2008) at a concentration of 20 pmoles, 3.5 μL of water and 1 μL of cDNA. The reaction was performed in a PrimeQ Real Time thermocycler (Techne) with the following parameters: 50 °C for 2 min, 95 °C for 5 min, 95 °C for 15 s, followed by 50 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. The value of the cycle threshold (Ct) for each sample was recorded.

Results from the biochemical and qPCR analyses were submitted to analysis of variance, with means separation using Tukey's HSD test ($\alpha = 0.05$). Data from the biochemical analysis were transformed using square root of Y + 1.0 using Sisvar software version 5.6 (Ferreira 2008).

Results

Biochemical analysis

Plants inoculated with the mild Pêra IAC isolate maintained under water deficit presented higher total protein content, with 3.85 mg protein/g of fresh matter in stressed plants and 2.50 mg protein/g in non-stressed plants (Table 1). The concentration of total proteins was higher in plants grafted on Rangpur lime (RL) under drought conditions, where the content total protein was 3.72 mg protein/g of fresh matter, while plants kept at field capacity showed 2.59 mg protein/g. In general, plants maintained under water deficit presented higher content of proteins, with 3.95 mg protein/g of fresh matter.

Table 1 Total protein content (mg of total protein/g of fresh matter) in sweet orange plants (*Citrus sinensis*) grafted on Swingle citrumelo or Rangpur lime infected with a mild (Pêra IAC) or severe (Forte Rolândia) isolate of citrus triteza virus and maintained at field capacity or under water deficit conditions

Treatments	Water regime	
	Field capacity	Water deficit
Forte Rolândia	3.22a*	3.87a
Pêra IAC	2.50b	3.85a
Control	3,32a	4.14a
Rangpur lime	2.59b	3.72a
Swingle citrumelo	3.44a	4.19a
Water Regime	3.01b	3.95a
C.V. 30.60%		

*Means followed by the same lowercase letters between water regimes do not differ from each other based on Tukey's HSD test ($\alpha = 0.05$)

Proline content did vary among treatments. In non-stressed plants there were no differences in the concentration of proline between the plants grafted on the rootstocks Swingle citrumelo (SC) and Rangpur lime (RL) (Table 2). However, in the stressed plants there were differences between the rootstocks used. The plants grafted on RL had a lower concentration of proline as compared to the SC rootstock, except for the plants that were inoculated with the CTV isolate Pêra IAC, in which there were no differences between the two rootstocks, showing 86.05 μmol proline/g of fresh matter in the SC rootstock and 65.66 μmol proline/g in the RL rootstock.

Table 2 Concentration of proline (μmol proline/g of fresh matter) in sweet orange (*Citrus sinensis*) plants grafted on Swingle citrumelo or Rangpur lime infected with a mild (Pêra IAC; PIAC) or severe (Forte Rolândia; FR) isolates of citrus tristeza virus and maintained at field capacity (FC) or under water deficit (WD) conditions

Treatments	Rootstock	
	Swingle citrumelo	Rangpur lime
FR/FC	9.36a*	8.69a
FR/WD	93.74a	58.95b
PIAC/FC	7.49a	3.61a
PIAC/WD	86.04a	65.66a
control/FC	6.70a	3.42a
control/WD	91.73a	66.37b
C.V.: 35.40%		

*Means followed by the same lowercase letters between rootstocks do not differ from each other based on Tukey's HSD test ($\alpha = 0.05$)

Table 3 Mean values of proline content (μmol proline/g fresh matter) in sweet orange (*Citrus sinensis*) plants grafted on Swingle citrumelo or Rangpur lime inoculated with mild (Pêra IAC) or severe (Forte Rolândia) isolates of citrus tristeza virus and maintained at field capacity or water deficit conditions

Treatments	Rootstock		Water Regime	
	Swingle citrumelo	Rangpur lime	Field capacity	Water deficit
Forte Rolândia	51.55a*	33.82b	9.03b*	76.35a
Pêra IAC	46.76a	34.64a	5.55b	75.85a
Control	49.21a	34.89a	5.05b	79.05a
CV: 35.40%				

*Means followed by the same lowercase letters between rootstocks do not differ from each other based on Tukey's HSD test ($\alpha = 0.05$)

The viral isolate and rootstock interaction showed that there were differences between plants inoculated with the CTV isolate Forte Rolândia when grafted on SC and RL. There was a greater content of proline in the samples when the graft was on SC rootstocks, with 51.55 μmol proline/g of fresh matter. For the CTV isolate Pêra IAC and for the control, there were no differences between the two rootstocks (Table 3). However, when the viral isolate and water regime interaction were evaluated, the plants maintained under drought conditions invariably had a higher concentration of proline (Table 2).

Plants grafted on SC and maintained under drought conditions had higher proline concentration compared to plants grafted on RL, and, in general, stressed plants showed higher proline concentration, with 77.08 μmol proline/g of fresh matter compared to non-stressed plants, which showed 6.54 μmol proline/g (Table 4).

Table 4 Concentration of proline (μmol proline/g of fresh matter) in sweet orange (*Citrus sinensis*) plants grafted on Swingle citrumelo or Rangpur lime and maintained at field capacity or under water deficit conditions

Treatments	Water Regime	
	Field capacity	Water deficit
Swingle citrumelo	7.85b*	90.50a
Rangpur lime	5.24b	63.66a
Water Regime	6.54b	77.08a
Treatments	Rootstock	
	Swingle citrumelo	Rangpur lime
Rootstock	49.18a	34.45b
CV: 35.40%		

*Means followed by the same lowercase letters between rootstocks or water regime do not differ from each other based on Tukey's HSD test ($\alpha = 0.05$)

There was no difference in catalase content among treatments or CTV strains.

Molecular analysis

All samples tested by qRT-PCR were positive for CTV, except for the negative controls (data not shown), and the amplification of the 3'-UTR region revealed differences between the treatments based on Tukey's HSD means separation (Table 5). Samples inoculated with the severe isolate, Forte Rolândia, and grafted on SC maintained at field capacity (non-stressed plants) had lower Ct values of the target gene sequence when compared to the Pêra IAC isolate under the same conditions, indicating a higher titer of virus. The water regime and viral isolate interaction showed difference between isolates in trees maintained at field capacity, with lower Ct values observed for the severe Forte Rolândia isolate, while the trees kept under drough conditions had no differences in Ct values.

Table 5 Mean Ct values obtained by qRT-PCR of sweet orange (*Citrus sinensis*) plants grafted on Swingle citrumelo (SC) or Rangpur lime (RL) inoculated with mild (Pêra IAC) or severe (Forte Rolândia) isolates of citrus tristeza virus and maintained at field capacity (FC) or under water deficit (WD) conditions

Treatments	Isolate	
	Pêra IAC	Forte Rolândia
SC/FC	25.72a	23.04b
SC/WD	25.31a	24.98a
RL/FC	26.00a	23.82a
RL/WD	24.25a	23.62a
FC	25.87a	23.43b
WD	24.79a	24.29a
Isolate	25.33a	23.86b
CV: 5.88%		

*Means followed by lowercase letters between rootstock and water regime do not differ among themselves by the Tukey test at 5% probability

In most cases the sweet orange samples inoculated with the Forte Rolândia isolate had a lower Ct value, indicating that this isolate existed at a higher titer in the plants maintained at field capacity compared to the Pêra IAC isolate. In this work, T_m values did not vary between mild and severe isolates, remaining at approximately 80 °C (data not shown).

Discussion

Among the various physiological mechanisms of the plant influenced by stress conditions, protein metabolism can be strongly altered, since protein synthesis is inhibited and the degradation of those available is accelerated (Larcher 2000). Some studies have shown that the stress condition imposed by lack of water, as well as by the presence of a pathogen, leads to the reduction of total soluble proteins (Oliveira Neto et al. 2006). In this work, plants inoculated with the Pêra IAC isolate kept under drought conditions presented higher total protein content. Those plants grafted on RL maintained under drought conditions also presented higher concentration of total proteins. Thus, there seems to be some contradiction between studies and it should be mentioned that host systems may behave differently. For example, RL has higher tolerance to drought and CTV (Pompeu-Junior 2005), and such condition may be related to the increase in total soluble protein concentration observed in this study. In addition, protein accumulation is used by some species of plants to tolerate dehydration (Claeys and Inze 2013).

Besides the accumulation of proteins, stressed plants also produce compatible solutes, such as proline, as a mechanism to tolerate the adverse condition to which they are exposed, responsible for the maintenance of turgescence, growth and photosynthesis (Morgan 1984). In this work, we observed that the water regime affected proline content. The use of different rootstocks influenced the proline content found in the stressed samples, as was observed in studies conducted by Carvalho et al. (2016). Some studies have demonstrated that drought-tolerant rootstocks accumulate a greater amount of proline (Ortuño et al. 2004; Zandalinas et al. 2016). The accumulation of this amino acid is not detrimental to cellular metabolism and, by increasing the osmotic pressure within the cells, it is able to maintain water absorption and the turgor pressure of the cells, which allows the continuity of physiological processes, even if at reduced levels (Marijuan and Bosch 2013).

Among the stressed plants there were differences between the rootstocks used, where sweet orange plants grafted under RL showed lower proline concentration, differing from the plants grafted under SC, which had higher values of this amino acid. The tolerance to drought induced by RL is associated to root conductivity and its growth, which also remobilizes the carbohydrate reserves for this organ (Medina and Machado 1998; Pedroso et al. 2014). The main adaptive response of plants to drought is the osmotic adjustment, which occurs due to the accumulation of solutes such as proline (Carvalho et al. 2016), and the rootstock may or may not influence the concentration of proline. In this work, in the non-stressed plants, the use of different rootstocks showed no differences in proline concentration, although it is known that SC plants are capable of producing and accumulating high levels of free proline in the leaves under conditions of growth and development (Nolte and Hanson 1997).

Catalase activity was not affected by the virus, drought or rootstock. Thus, the results of our study differ from those obtained by Pérez-Clemente et al. (2015), who verified that when a plant is infected by CTV there is a reduction in catalase activity. However, our results agree with data presented by Dória et al. (2015), who found that catalase activity was not different between healthy and CTV-infected plants. According to Hančević et al. (2018), infection by different CTV isolates does not interfere in the activity of this enzyme. Biochemical responses in plant defense against pathogens are performed by several enzymes, not only by catalase, and these can reduce the damage caused by the pathogen or prevent its progression in host plant tissues (Resende et al. 2003). It is also known that the concentration of catalase can increase or decrease according to the time the plant undergoes stress (Hussain et al. 2018). In addition, the absence of differences in the catalase content between stressed and non-stressed plants may be related to the fact that proline can modify the activity of antioxidant enzymes, as observed in Swingle citrumelo plants under normal water supply conditions and during water deficit (Campos et al. 2011).

CTV was found in all samples inoculated in this study, although titer varied. Under field capacity conditions, the severe isolate Forte Rolândia reached a higher concentration than the protective isolate Pêra IAC. In addition, the Forte Rolândia isolate also showed a higher viral titer when inoculated in SC plants maintained at field capacity. CTV isolates behave differently in different citrus hosts (Laino et al. 2016). In the quantitative detection performed by Bertolini et al. (2008), Ct values varied among the different isolates tested, and the lowest Ct values were verified for the severe isolates, as observed in this study. In addition, the combination of the rootstock as well as the type of material from which the viral RNA was extracted, such as petioles or leaf veins, also influence the viral concentration (Ruiz-Ruiz et al. 2007).

In general, the water stress condition did not influence the viral replication process. However, under normal growth and development conditions (field capacity), plants infected with the severe isolate had a higher viral concentration than those infected with the protective isolate. It should be taken into account that these plants were kept at the ideal temperature for viral replication (20 °C) and, according to Corazza et al. (2012), severe CTV isolates have greater expression in milder temperatures.

Differently from Ct values, which revealed differences between severe and mild isolates, no difference was observed in the dissociation temperature (T_m) of the primers of the target sequence. According to Ruiz-Ruiz et al. (2007) this value may

be a tool to differentiate mild and severe isolates: the T_m of severe isolates is supposed to be considerably higher than that of mild isolates. However, in this work, T_m values did not vary between mild and severe isolates. The variation in T_m values is associated with the amplicon size and GC content present in the sample (Ririe et al. 1997), which justifies the results found in this work, since the isolates had the same amplicon size and very similar GC contents.

This work confirmed differences in the evaluation of total protein content and proline, with greater accumulation in plants maintained under water deficit. There was no effect of drought on the population of viral isolates, but the plants held at field capacity and inoculated with the severe isolate had a higher viral titer. In general, under conditions of these experiments, Swingle citrumelo was confirmed to behave better under water stress.

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