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Citrus sudden death-associated virus (CSDaV) and citrus tristeza virus (CTV) in eleven rootstocks for 'Valencia' sweet orange

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Abstract – Citrus sudden death (CSD) is a highly destructive disease and has caused the eradication of millions of trees in southern Brazil within the last 15 years. In spite of the exact cause of CSD has not been determined, evidences have shown that this disease can be transmitted by biotic vectors. Disease incidence in sweet orange scions is related to the rootstock, and the combination with 'Rangpur' lime is the most affected. On the other hand, there are evidences of a relation between CSD affected trees and the presence of the *Citrus sudden death associated virus* (CSDaV) and/or *Citrus tristeza virus* (CTV). Based on such information, this study has been carried out to determine the presence of CSDaV and CTV, and the association between each other in eleven rootstocks for 'Valencia' sweet orange. The results presented herein showed differences related to the presence of CSDaV and CTV in different rootstocks for 'Valencia' sweet orange and no relation between the presence of CSDaV and CTV.

Index terms: *Marafivirus*, *Poncirus trifoliata*, symptoms, transmission.

Citrus sudden death-associated virus (CSDaV) e citrus tristeza vírus (CTV) em onze porta-enxertos para laranja 'Valencia'

Resumo – A morte súbita dos citros (MSC) é uma doença de alto impacto destrutivo e tem levado à erradicação de milhões de plantas de laranja-doce na região Sudeste do Brasil, nos últimos 15 anos. Embora o agente causal da MSC ainda não tenha sido determinado, evidências demonstraram que esta doença pode ser transmitida por um vetor biótico. A incidência de MSC em cultivares de laranja-doce está relacionada ao porta-enxerto, sendo que a combinação com limoeiro 'Cravo' é a mais afetada. Por outro lado, há evidências de relação entre plantas sintomáticas para MSC e a presença de um vírus, denominado *Citrus sudden death associated virus* (CSDaV) e/ou com *Citrus tristeza virus* (CTV). Com base nessas informações, o objetivo deste estudo foi determinar a presença de CSDaV e CTV e a associação entre eles, em onze porta-enxertos para laranjeira 'Valencia'. Os resultados apresentados aqui sugerem diferenças relacionadas à presença de CSDaV e CTV em diferentes porta-enxertos para laranja 'Valencia', não havendo relação entre a presença de CSDaV e CTV.

Termos para indexação: *Marafivirus*, *Poncirus trifoliata*, sintomas, transmissão.

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Introduction

Citrus sudden death (CSD) was first reported in 2001 throughout 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] groves grafted onto 'Rangpur' lime, in Minas Gerais State, Brazil (GIMENES-FERNANDES; BASSANEZI, 2001; MÜLLER et al., 2002). CSD was the cause for the eradication of nearly four million trees between 2001 and 2006 corresponding to 2% of the population of sweet orange trees in São Paulo and Minas Gerais States (YAMAMOTO et al., 2011).

CSD incidence in sweet orange scions is related to the rootstock. 'Rangpur' lime (*Citrus limonia* Osbeck), 'Volkamer' lemon [*Citrus ×volkameriana* (Risso) V. Ten. & Pasq.] and 'Rough' lemon (*Citrus jambhiri* Lush.) are susceptible to CSD. Rootstocks such as 'Cleopatra' mandarin (*Citrus reshni* hort. ex Tanaka), 'Sunki' mandarin (*Citrus sunki* hort. ex Tanaka), 'Swingle' citrumelo [*Citrus paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.] and trifoliata [*Poncirus trifoliata* (L.) Raf.] are considered resistant to this disease (MÜLLER et al., 2005; YAMAMOTO et al., 2011).

CSD can be transmitted by grafting (YAMAMOTO et al., 2011), supporting the evidence that the causal agent is biotic, considering that the spatial and temporal patterns of disease development were similar to those reported for citrus tristeza dissemination (BASSANEZI et al., 2003), and the successful transmission by unknown aerial biotic vectors (YAMAMOTO et al., 2011). In 99.7% of CSD affected trees, a virus tentatively belonging to the genus *Marafivirus,* family *Tymoviridae* was reported, and denominated by Maccheroni et al. (2005) *Citrus sudden death associated virus* (CSDaV). This same virus was found in aphids, suggesting that these insects may play an important role in the disease transmission. The association between *Citrus tristeza virus* (CTV) and CSDaV as the causal agent of CSD has been considered (ROMÁN et al., 2004).

Based on these findings, the aim of this current study was to determine the presence of CSDaV and CTV, and association between each other, in 'Valencia' sweet orange plants grafted on eleven rootstocks**.**

Materials and Methods

The experiment was carried out in the northern region of São Paulo State, Brazil. The experimental site is located at latitude 20 ° 19 ' 39 " S and longitude 48 ° 41' 16" W. The climate, according to Köppen classification, is Aw (tropical, rainy with dry winter), the minimum average temperature is 17.0°C and the maximum is 30.7°C, the pluviometric precipitation range is 1430 mm per year (CEPAGRI, 2014). This region is endemic for CSD.

'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] plants were grafted onto two accesses of 'Rangpur' lime (*C. limonia* Osbeck) ("CNPMF 03" and "Santa Cruz"), three accesses of tangerine (*C. reticulata* Blanco) ('Malvásio SRA 115', 'East India SRA 414' and 'C-54-4-4 SRA 337'), 'À Peau Lisse SRA 267' tangerine (*C. deliciosa* Ten.), the hybrids 'Sunki' mandarin x 'Benecke' *Poncirus trifoliata* [*C. sunki* (Hayata) hort. ex Tanaka x *Poncirus trifoliata* (L.) Raf.], 'C-13' "S" citrange [*C. sinensis* (L.) Osbeck x *P. trifoliata* (L.) Raf.], 'Cleópatra' mandarin x 'Rubidoux' *Poncirus trifoliata* [*C. reshni* hort. ex Tanaka x *P. trifoliata* (L.) Raf.], 'Sunki' mandarin x 'English' *Poncirus trifoliata* [*C. sunki* (Hayata) hort. ex Tanaka x *P. trifoliata* (L.) Raf.] and the somatic hybrid 'Rohde Red' 'Valencia' sweet orange + 'Volkamer' lemon [*C. sinensis* (L). Osbeck + *C. volkameriana*]. All experimental plants were produced with the use of micro grafted and CTV pre-immunized 'Valencia' sweet orange buds. Plant material was planted in March 2007, in a 6.0 m x 2.5 m spacing, and cultivated until 2014 without irrigation.

Experiment design followed a randomized block design, with eleven treatments (rootstocks), three replications (blocks) and five plants per plot, in a total of 165 plants. This field experiment comprised 15 repetitions of each rootstock and the whole experiment was carried out once, between 2007 and 2014.

All 'Valencia' sweet orange trees were visually inspected for CSD symptoms once a year, between 2010 and 2013, searching for CSD symptoms such as opaque and general leaf chlorosis, leaf drop, apical shoot death and scarce shoots (BASSANEZI et al., 2003), comprising four evaluations of canopy symptoms. Moreover, in November 2013, the detection of the most typical CSD symptom, i.e., the presence of a yellow-stained layer and/or a thickness in the bark in the rootstock (bellow the graft union), contrasting with the scion bark lighter was also carried out (GIMENES-FERNANDES; BASSANEZI, 2001; YAMAMOTO et al., 2011). The partial bark removal in the grafting zone (five centimeters below and five centimeters above graft union) was carried out in all trees to detect the bark rootstock yellowing. All trees were also photographed for further observations and possible correlation studies between the presence of CSDaV and CTV and tree symptoms.

The bark samples were stored at –80 °C for four weeks. Then, the samples were washed and dried. Each bark sample was sliced into small sections with a razor blade, and 500 mg of this material were used for total RNA extraction after grinding in liquid N_2. Total RNA was extracted with Trizol LS reagent (Invitrogen) according to the manufacturer's instructions.

The final RNA pellet was suspended in 30 µL of sterile water treated with 0.1% diethyl pyrocarbonate (DEPC, Sigma), and was either immediately used or stored at –20°C. The cDNA was synthesized with Improm II reverse transcriptase (1 µL, Promega), 1 µL total RNA,

1 µL RNase OUT (Invitrogen), 0.5 µM random hexamer primer, 0.2 mM dNTPs, 1× Improm II buffer, 3 mM $MgCl_2$ in 20 µL final volume.

For CSDaV and CTV detection, four PCR were performed, two single reactions for CSDaV and two duplex reactions for CSDaV and CTV. For evaluation purposes, we considered positive samples those with CSDaV or CTV presence in, at least, one PCR.

In analyses for CSDaV detection, PCR mix included 1.0 µL cDNA, 1× Platinum *Taq* buffer, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen) in a 20 µl final volume of reaction mixture. The detection of a 622-bp amplified from the CSDaV was performed using primers Com_F (5′ATGGTCTCAGGCGACGACTCCCT3′) and Com_R (5′GTGAGGATGGGAGCAGAGGAAC3′) according to Yamamoto et al. (2011). The PCR product was electrophoresed in 1.2% agarose gel at 1× TAE and visualized under UV light after ethidium-bromide staining.

For the duplex PCR, the mix included 1.0 µL cDNA, 1× Phire buffer, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, and 0.4 µL of Phire hot start DNA polymerase (Finnzymes) in a final reaction mixture of 20 µL. The detection of a 246-bp amplified from the CSDaV was performed using primers C1_For (5′CCGCTGTCACCATTGCTTCCAG3′) and Geno_Rev (5′GGGAACTCATTGTGGAACCAGTCA3′) (YAMAMOTO et al., 2011). The detection of a 600-bp amplified from the CTV was performed using primers CN 119 e CN 120 (ROY; RAMACHANDRAN, 2002) according to Yamamoto et al. (2011). The PCR product was electrophoresed in 1.2% agarose gel at 1× TAE and visualized under UV light after ethidium-bromide staining.

Data regarding CSDaV and CTV detections (absence or presence) were analyzed by Fischer's exact test and Spearman's correlation (CAMPOS, 1983).

Results and Discussion

Typical CSD scion symptoms (shoots and leaves) and typical CSD symptoms in the grafting zone were not detected in any 'Valencia' sweet orange trees grafted onto eleven rootstocks. Some yellow-staining in the rootstock bellow graft union was registered in just one tree grafted onto 'Rohde Red' 'Valencia' sweet orange + 'Volkamer' lemon somatic hybrid rootstock, but it was not considered a typical CSD symptom.

According to the Fischer's exact test (frequency of CSDaV presence and absence) the rootstocks were divided into three groups, regarding the percentage of CSDaV presence (Table 1). Group 1 comprised the rootstocks that showed lower percentage of CSDaV positive plants; Group 2 was composed of rootstocks that showed intermediate percentage of CSDaV positive plants. Finally, Group 3 comprised those rootstocks that had higher percentage of CSDaV positive plants. CSDaV detection (YAMAMOTO et al., 2011) was targeted to two loci in the virus genome, helicase and RNA-dependent RNA polymerase/coat protein region (MACCHERONI et al., 2005) and those regions where characterized as of higher and lower nucleotide variability (MATSUMURA et al., 2016), providing enough support for the detection techniques employed in this work.

"Santa Cruz" 'Rangpur' lime pooled between rootstocks with lower percentage of CSDaV presence (Table 1). Although 'Rangpur' lime can be considered susceptible to CSD (BOVÉ; AYRES, 2007), "Santa Cruz" 'Rangpur' lime differed to "CNPMF 03" 'Rangpur' lime (classified as intermediate). On the other hand, the somatic hybrid 'Rohde Red' 'Valencia' sweet orange + 'Volkamer' lemon pooled between rootstocks with higher percentage of CSDaV and CTV positive plants (Table 1 and 2).

The same criterion utilized for CSDaV evaluation was applied for CTV. In spite of host reaction to CTV being dependent on scion/rootstock interaction, the main objective in this study was to evaluate the interaction between CTV/CSDaV, and not to determine the scion/rootstock resistance or tolerance to CTV.

Regarding CTV detection in experimental plants, different groups comprised the main differences among rootstocks (Table 2). Both CSDaV and CTV were divided into three groups, although there were differences related to rootstocks number into each group.

"Santa Cruz" 'Rangpur' lime and 'C-54-4-4 SRA 337' tangerine were classified as rootstocks with low CTV presence, 'Sunki' mandarin x 'English' *P. trifoliata* and 'À Peau Lisse SRA 267' were classified as rootstocks with intermediate percentage of CTV presence, 'Rohde Red' 'Valencia' sweet orange plus 'Volkamer' lemon was classified as rootstocks with the highest percentage of virus presence, both for CSDaV and CTV.

Despite the similarity between some rootstocks related to CSDaV and CTV, there was not significant correlation between the presence of CSDaV and CTV according to the Sperman's correlation analysis (P = 0.9798 and correlation coefficient = 0.00459). This information suggests that it is not possible, at least in this work, to assume that an association between CSDaV and CTV is the main cause of CSD, as suggested by previous research (MACCHERONI et al., 2005; ROMÁN et al., 2004). Moreover, CTV is present in most of the Brazilian citrus areas (MÜLLER et al., 2005). Furthermore, as previously mentioned, all nursery plants used in this experiment were produced using micro grafted and CTV pre-immunized buds.

It has been suggested that the CSD agent is not a natural variant of CTV, because CTV variant analyses in *Aphis spiraecola* and in *Toxoptera citricida* did not find a pattern between symptomatic and asymptomatic plants (LOEZA-KUK et al., 2008). Another hypothesis could be

that the cause of CSD is related to mixed infections with two or more CTV variants, or even with the interaction among CTV variants (GOMES et al., 2008). Cantú et al. (2008) found that plants affected by CSD had chitinase and miraculin proteins in much lower levels than those in healthy trees. 'Valencia' sweet orange grafted onto 'Volkamer' lemon with initial CDS scion symptoms also had symptoms in the rootstock bark (ROMÁN et al., 2004). In the present study, the somatic hybrid 'Rohde Red' 'Valencia' sweet orange + 'Volkamer' did not show CSD symptoms on the scion, in spite some yellow-staining in the rootstock.

Regarding the results obtained in this study time (seven years after the field transplanting), it is evident the need of further evaluations, because no CSD symptoms was observed on the scion or in the rootstock so far. The differences discussed herein can support future evaluations related to the rootstocks involved in this present study.

Table 1. Percentage of CSDaV positive rootstocks determined by RT-PCR in 'Valencia' sweet orange grafted onto eleven rootstocks, November 2014.

Rootstock/Group	CSDaV
'Cleópatra' mandarin x 'Rubidoux' *P. trifoliata*	57.14
'Sunki' mandarin x 'Benecke'*P. trifoliata*	63.64
"Santa Cruz" 'Rangpur' lime	61.54
'C-54-4-4 SRA 337' tangerine	66.67
Group I (*P*)*	0.9790
'Sunki' mandarin x 'English' *P. trifoliata*	73.33
'À Peau Lisse SRA 267' tangerine	73.33
'East India SRA 414' tangerine	73.33
"CNPMF 03" 'Rangpur' lime	80.00
Group II (*P*)*	1.000
'C-13' "S" citrange	85.71
'Malvasio SRA 115' tangerine	86.67
'Rohde Red' 'Valencia' sweet orange plus 'Volkamer' lemon	92.86
Group III (*P*)*	1.000
Between groups (*P*)**	0.0131

**P*<0.05 values between same rootstock group indicate significance at 5% probability by Fisher's exact test. ** *P*<0.05 values between groups indicate significance at 5% probability by Fisher's exact test.

Table 2. Percentage of CTV positive rootstocks determined by RT-PCR in 'Valencia' sweet orange grafted onto eleven rootstocks, November 2014.

Rootstock/Group	CTV
"Santa Cruz" 'Rangpur' lime	46.15
'C-54-4-4 SRA 337' tangerine	46.15
'C-13' "S" citrange	50.00
Group I (*P*)*	1.000
'Malvasio SRA 115' tangerine	60.00
'Sunki' mandarin x 'Benecke'*P. trifoliata*	63.64
'À Peau Lisse SRA 267' tangerine	66.67
'Cleópatra' mandarin x 'Rubidoux' *P. trifoliata*	71.43
'Sunki' mandarin x 'English' *P. trifoliata*	73.33
Group II (*P*)*	0.9639
'Rohde Red' 'Valencia' sweet orange plus 'Volkamer' lemon	85.71
"CNPMF 03" 'Rangpur' lime	93.33
'East India SRA 414' tangerine	93.33
Group III (*P*)*	0.6751
Between groups (*P*)**	<0.0001

P<0.05 values between same rootstock group indicate significance at 5% probability by Fisher's exact test. ** *P*<0.05 values between groups indicate significance at 5% probability by Fisher's exact test.

Conclusions

The presence of CSDaV and CTV was distinct among different rootstocks for 'Valencia' sweet orange. Among the 11 scion/rootstocks combinations, those with 'Cleópatra' mandarin x 'Rubidoux' *P. trifoliata*, 'Sunki' mandarin x 'Benecke' *P. trifoliata*, "Santa Cruz" 'Rangpur' lime, and 'C-54-4-4 SRA 337' tangerine rootstocks had lower percentage of CSDaV comparing with the others in the group. On the other hand, the lowest percentage of CTV positive rootstocks were the combinations on "Santa Cruz" 'Rangpur' lime, 'C-54-4-4 SRA 337' tangerine, and 'C-13' "S" citrange.

No relation between the presence of CSDaV and CTV could be determined.

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