Tropical Plant Pathology, vol. 35, 5, 303-309 (2010)

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SHORT COMMUNICATION / *COMUNICAÇÃO*

Viroid species associated with the bark-cracking phenotype of 'Tahiti' acid lime in the State of São Paulo, Brazil

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ABSTRACT

Viroids have been used as "graft transmissible dwarfing agents" (GTDA) in several countries, mainly to reduce growth of citrus trees, thus increasing their density in orchards. In the State of São Paulo, Brazil, plants of the acid lime 'Tahiti' are usually grafted with a complex of GTDA, presumably viroids. The aim of the present work was the identification and molecular characterization of the viroids infecting trees of acid lime 'Tahiti' displaying "Quebra galho" (bark-cracking). Viroids were identified and characterized by biological indexing in 'Etrog' citron, Northern-blot hybridization, RT-PCR, cloning and complete sequencing of the RNA genomes. *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus dwarfing viroid* (CDVd) were found in different combinations. Although we have not been able to infer a direct relationship between the agronomical performance and symptom severity with the presence of a specific viroid or viroid combination, the differences in the severity of "Quebra-galho" symptoms among different trees is probably associated with the presence (or absence) of CEVd, with its interaction with other viroids perhaps determining the different phenotypes observed in the field.
Key words: Exocortis, Cachexia, Xyloporosis, *Pospiviroidae, Pospiviroid,* Citrus dwarfing.

RESUMO

Espécies de viróides associadas a caneluras nos ramos em limeira ácida 'Tahiti' no Estado de São Paulo, Brasil

Os viroides têm sido empregados em diversos países como agentes indutores de nanismo transmissíveis por enxertia (*"graft transmissible dwarfing agents"*, GTDA), principalmente visando à diminuição do porte das plantas, o que permite o adensamento do pomar. No Estado de São Paulo, Brasil, plantas de limeira ácida 'Tahiti' são normalmente enxertadas com gemas contendo GTDA, presumivelmente viroides. Portanto, o presente trabalho visou à identificação e caracterização molecular dos viroides presentes em plantas de limeira ácida 'Tahiti' apresentando caneluras nos ramos (*bark-cracking*), conhecido como "Quebra galho". Os viroides foram identificados e caracterizados por meio de indexação biológica em cidreira 'Etrog', hibridização por *Northern Blot,* e RT-PCR seguida de clonagem e sequenciamento completo dos genomas. Identificaram-se *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) e *Citrus dwarfing viroid* (CDVd) em diferentes combinações. Embora não tenha havido associação direta entre desempenho agronômico e severidade dos sintomas com a presença de um determinado viroide ou combinação de viroides, as diferenças na severidade dos sintomas de "Quebra-galho" está associada com a presença (ou ausência) do CEVd, e as interações do CEVd com outros viroides deve ser, provavelmente, determinante nas diferenças fenotípicas observadas em campo.
Palavras-chave: Exocorte, Cachexia, Xiloporose, *Pospiviroidae, Pospiviroid*, nanismo.

Viroids, the smallest known plant pathogens, consist of a non-protein-coding, small (246-401 nucleotides, nt), circular, single-stranded RNA that replicates autonomously when inoculated in their host plants. Currently, viroids are classified according to their molecular and biological properties into two families, *Pospiviroidae* and *Avsunviroidae,* with five and three genera, respectively (see for reviews Daròs et al., 2006; Flores & Owens, 2008; Ding, 2009). All citrus viroids belong to the family *Pospiviroidae,* the species of which present a central conserved region (CCR), replicate in the nucleus of infected cells through an asymmetric rolling-circle mechanism, and lack hammerhead ribozymes. To date, five natural citrus viroid species have been described: *Citrus exocortis viroid* (CEVd, genus *Pospiviroid*), *Hop stunt viroid* (HSVd, genus *Hostuviroid*), *Citrus viroid IV* (CVd-IV, recently renamed *Citrus bark cracking viroid*, CBCVd, genus *Cocadviroid*), and *Citrus bent leaf viroid* (CBLVd) and *Citrus viroid III* (CVd-III, recently renamed *Citrus dwarfing viroid*, CDVd) of the genus *Apscaviroid* (see for reviews Flores & Owens, 2008; Eiras et al., 2009). In addition, *Citrus viroid V* (CVd-V) (Serra et al., 2008a,b) and *Citrus viroid VI* (formerly

known as *Citrus viroid original source,* CVd-OS) (Ito et al., 2001), have been recently accepted as new species of the genus *Apscaviroid*.

Citrus viroids are graft-transmitted and their dissemination occurs mainly by propagation of contaminated bud-wood material. In nature, they are found infecting different species of the genus *Citrus* and closely-related genera, and are usually present as complex mixtures co-infecting the same plant (Durán-Vila et al., 1986, 1988; Durán-Vila & Semancik 2003; Barbosa et al., 2005). Two well-known viroid diseases of citrus are exocortis, induced by CEVd (Semancik & Weathers, 1972a; Semancik & Weathers, 1972b) and cachexia, induced by some specific variants of HSVd (Semancik et al., 1988; Reanwarakom & Semancik, 1999; Serra et al., 2008c). However, viroids do not incite conspicuous symptoms in some citrus species, apart from affecting moderately the tree size and crop yield (Vernière et al., 2004). Viroid control is based on preventive measures, namely the use of viroid-free bud-wood as propagation material and adequate indexing procedures.

In Brazil, citrus represents one of the most important crops with a total area estimated at 1.5 million hectares (ha) and 19 million tons in production. The country is the world's largest exporter of orange juice, generating 1.5 billion dollars per year (Mattos Junior et al., 2005). The acid lime 'Tahiti' [*Citrus latifolia* (Yu. Tanaka) Tanaka] occupies an area of 47085 ha with an average productivity estimated at 12.3 ton/ha, with the State of São Paulo accounting for 82% of the total production. Exportation of the Brazilian acid lime 'Tahiti' reached 42359 tons in 2008 (Agrianual, 2009).

Two kinds of clones of the acid lime 'Tahiti' are cultivated in the State of São Paulo: i. the IAC-5 or Peruano, a nucelar viroid-free clone, and ii. the "Quebra-galho" clones (Salibe & Moreira, 1965) infected by viroids. Although the "Quebra-galho" clones present a non-uniform field behavior with high plant size variability in the orchards and a limited productive life, they are preferred by growers because the small tree size permits high density planting and facilitates harvesting (Silva, 2007). The name "Quebra-galho" derives from the bark-cracking symptom, resulting in brittle branches, probably associated with viroid infection.

The present work aimed to identify and characterize the viroid species infecting trees of acid lime 'Tahiti', grafted on Rangpur lime (*Citrus limonia* Osbek) as rootstock, displaying "Quebra-galho" selected in the State of São Paulo by Silva (2007) because of their different agronomical properties: productivity, fruit quality and tree size.

Six 'Tahiti' acid lime trees graft-inoculated with material from different "Quebra-galho" clones (1.4, 4.1, 4.6, 7.4, 9.5 and 10.4) were selected from orchards in Catanduva region, State of São Paulo. Symptoms in acid lime 'Tahiti' plants grown under field conditions were classified as: i. "Mild", with few and small bark-cracks, ii. "Moderate", with frequent bark-cracks, and iii. "Severe", displaying easily visible and extended "Quebra-galho" bark-cracking on the canopy (Figure 1). This symptom classification was created based on previous observations of 80 'Tahiti' trees in different orchards in the State of São Paulo (Silva, 2007). For viroid indexing and bio-amplification, citrons (*Citrus medica* L.) 'Etrog Arizona S1', propagated on Rangpur lime as a rootstock, were grafted with buds from the medial portion of the selected acid lime trees (Roistacher et al., 1991). Plants were maintained in a greenhouse with a temperature ranging from 28 to 36°C and, 60 days after grafting, citron leaves were collected for RNA analysis. Grafted citrons reacted by expressing strong leaf curling or no symptoms at all.

Young leaves (10 g) of acid lime 'Tahiti' and 'Etrog' citron were ground with liquid nitrogen and homogenized in the presence of a mixture of water-saturated phenol and extraction buffer (125 mM Tris-HCl, pH 9.0, 0.75% SDS, 15 mM EDTA, 100 mM 2-mercaptoethanol). After extraction, total RNA was fractionated by chromatography on non-ionic cellulose CF11 (Whatman), recovered by ethanol precipitation, resuspended in water (Flores et al., 1985) and subjected

FIGURE 1 - Different severity of bark-cracking symptoms on acid lime 'Tahiti' clone "Quebra-galho". **A.** Mild symptoms; **B.** Moderate symptoms; **C.** Severe symptoms.

to polysaccharide extraction (Bellamy & Ralph, 1968). Aliquots of the RNA preparations were electrophoresed in a non-denaturing 5% polyacrylamide gel that was stained with ethidium bromide and a segment of this gel, delimited by *Avocado sunblotch viroid* (ASBVd, 246 nt) and CEVd (371 nt) RNAs, was cut and applied onto a second denaturing 5% polyacrylamide gel that was stained with silver nitrate (Flores *et al*., 1985). Other RNA aliquots separated by native or denaturing PAGE were electrotransferred to positively-charged nylon membranes (Roche) and immobilized by UV cross-linking. Linear plasmids (*pBluescript II KS*+ or pGEM-T vectors) containing distinct citrus viroid cDNAs were used as templates for synthesis of full-length riboprobes complementary to the plus strands of each citrus viroid (CEVd, HSVd, CBLVd, CDVd, CBCVd and CVd-V) using T7, T3 or SP6 RNA polymerases in the presence of [α-^{32}P]UTP (400 Ci/mmol). Pre-hybridization (at 70ºC for 2 h) and hybridization (at 70ºC overnight) were performed in the presence of hybridization solution (50% formamide, 65 mM sodium phosphate pH 6.5, 60 mM NaCl, 7.5 mM sodium citrate, 10 mM EDTA, 1% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 100 µg/mL of denatured salmon sperm DNA). The membranes were washed three times in 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate) with 0.1% SDS at room temperature for 10 min, once in 0.1X SSC with 0.1% SDS at 55ºC for 15 min, and revealed by autoradiography.

Samples 4.6 and 10.4 for CEVd and HSVd, and 4.6 and 7.4 for CDVd, were selected for RT-PCR amplification and cloning. The couples of adjacent primers derived from each viroid CCR were: i. CEVd, PI (complementary, c) 5'-GGGGATCCCTGAAGGACTTCT-3' and PII (homologous, h) 5'-GGGGAAACCTGGAGGAAG-3'; ii. HSVd, PIII (c) 5'-ACTCTTCTCAGAATCCAGCGAG-3' and PIV (h) 5'-TGCCCCGGGGCTCCTTTCTCAGGT-3'; and iii. CDVd, PV (c) 5'-TTCGTCGACGACGACAGGTA-3' and PVI (h) 5' GGCAGCTAAGTTGGTGACGC-3'. The cDNA was synthesized using 0.25 µg of total RNA and 5 pmol of each complementary primer, with 50 U of M-MuLV reverse transcriptase (RT) (Fermentas), 1 mM dNTPs and 10 U of RNase inhibitor in the RT buffer recommended by the supplier. The reaction mixture was incubated at 42ºC for 45 min, 50ºC for 10 min and 60ºC for 5 min. PCR was carried out in 50 µL using 1 µL of the cDNA reaction mixture, 2.5 U of *Tth* DNA polymerase (Biotools), 50 pmol of each primer, 0.2 mM dNTPs and the buffer recommended by the supplier. Samples were denatured at 94°C for 2 min and the amplification profile consisted of 30 cycles of 40 s at 94ºC, 30 s at 60ºC, and 2 min at 72°C with a final extension step at 72°C for 10 min. The full-length amplification products (371 bp for CEVd, 297 bp for HSVd and 294 bp for CDVd) were cloned, sequenced, aligned and compared with other viroid sequences in the "Subviral RNA Database" (http://subviral.med.uottawa.ca/cgi-bin/accueil.cgi?typeRNA=1). Secondary structure analysis was done with the Mfold program applied to circular RNA (Zuker, 1989).

To identify and characterize the viroid species infecting acid lime 'Tahiti' trees of clones "Quebra-galho", plants of 'Etrog' citron were employed for viroid bio-amplification, since all citrus viroids do accumulate at detectable titers in this host and also for biological indexing. Leaf curling on 'Etrog' citron was observed when inoculated with material from 1.4, 4.1, 4.6 and 10.4 sources, while sources 7.4 and 9.5 did not incite any reaction (Table 1). In the field, the corresponding acid lime 'Tahiti' trees exhibited different degrees of "Quebra-galho" (bark-cracking) in the canopy: symptoms were mild in plants 7.4, 9.5 and 10.4, moderate in 1.4 and 4.1, and severe in 4.6 (Table 1).

RNA preparations from acid lime 'Tahiti' and 'Etrog' citron were analyzed by PAGE. Viroids could be detected by silver staining only after bio-amplification in 'Etrog' citron (data not shown), while they were readily detected in 'Etrog' citron and acid lime 'Tahiti' by Northern Blot hybridization with ^{32}P-labelled riboprobes. Attempts to use PAGE analysis resulted in unreliable results because in many citrus species, including acid lime 'Tahiti', viroids do not accumulate at high enough titers to be detected (Murcia et al., 2009). Although specific probes for the six citrus viroids were used, only three (CEVd, HSVd and CDVd) were found in different combinations infecting acid lime 'Tahiti' plants (data not shown). These results were confirmed with the corresponding RNA preparations from 'Etrog' citron (Figure 2). CEVd was present in samples 1.4, 4.1, 4.6 and 10.4, HSVd in the sample 10.4, and CDVd in samples 4.1, 4.6, 7.4, 9.5 and 10.4. Table 1 summarizes results from biological and molecular indexing.

TABLE 1 - Reaction observed in the acid lime 'Tahiti' and in the indicator 'Etrog' citron, and viroids detected in the latter

Sample	Reaction on 'Tahiti' field trees	Reaction on 'Etrog' citron	Northern Blot on 'Etrog' citron
1.4	Moderate	Epinasty	CEVd
4.1	Moderate	Epinasty	CEVd, CDVd
4.6	Severe	Epinasty	CEVd, CDVd
7.4	Mild	No reaction	CDVd
9.5	Mild	No reaction	CDVd
10.4	Mild	Epinasty	CEVd, HSVd, CDVd

To further corroborate these results, infected samples were selected for each viroid according to hybridization data and subjected to RT-PCR amplification, cloning and sequencing. Four clones from samples 4.6 and 10.4 for CEVd, four clones from sample 10.4 for HSVd, and six clones from samples 4.6 and 7.4 for CDVd were sequenced. These clones were selected because they were identified in plants infected by the same viroids but displaying different symptoms (Table 1). Comparisons revealed a high degree of sequence identity with variants of CEVd (93 to 99%), HSVd (97 to 99%) and CDVd (99 to 100%) deposited in databases, with the main difference being observed among CEVd clones: one from sample 4.6 was 372-nt in length, whereas a 371-nt clone was isolated from sample 10.4 as a result of the deletion of a U between positions 84 and 85 in the former, together with an insertion of an A in position 76 and a deletion of a U between positions 129-130, with three substitutions at positions 364 (G→A), 309 (U→C) and 300 (A→U) (Figure 3). These comparisons were done using the variant CEVd-C with 371-nt in length as reference (Gross et al., 1982). Despite their high sequence identity, CEVd clones presented some variations indicative of a mixture of sequence variants (Gandía et al. 2005). A similar situation has been described for HSVd (Palacio-Bielsa et al., 2004). All the HSVd clones here sequenced lacked the cachexia-associated motif and the corresponding sources did not incite symptoms when inoculated on the indicator 'Parson's Special' mandarin (data not shown). These observations are in accordance with previous results obtained by Targon et al. (2005) and Silva (2007) in their analyses of 'Tahiti' plants from the State of São Paulo: only non-cachexia HSVd isolates were identified. Finally, the six CDVd clones here sequenced showed high sequence conservation, in accordance with previous data (Owens et al., 1999).

A direct relationship between the agronomical performance of field trees and the presence of a specific viroid or a viroid combination was not observed: apart from inducing tree dwarfing, CDVd infection of plants 7.4 and 9.5 (Table 1) incited just mild symptoms on the canopy of the acid lime 'Tahiti'. Silva (2007) analyzed the fruit quality and production of the same trees and did not observe any significant reduction. These results confirm that CDVd does not cause important phenotypic alterations and, therefore, that 7.4 and 9.5 could be interesting sources for citrus dwarfing (Owens et al., 1999). In contrast, the presence of CEVd and CDVd in samples 4.1 and 4.6 was associated with moderate and severe bark-cracking, respectively, and the three viroids (CEVd, HSVd and CDVd) in sample 10.4 with mild symptoms (Table 1). Despite displaying severe symptoms, tree 4.6 performed agronomically better regarding fruit quality and production (Silva, 2007).

There are few data on the effects incited by individual viroids in citrus grown under field conditions. In a field assay conducted for a 12-year period, Vernière et al. (2004) evaluated the effect of inoculating separately each citrus viroid (CEVd, HSVd, CBLVd, CDVd and CVd-IV) on symptom expression of 'Clementine' trees grafted on 'Trifoliate' orange. Their results indicate that citrus viroids can elicit a broad range of symptoms, from the severe scaling induced by CEVd and bark-cracking induced by CEVd, HSVd and CVd-IV, to minor effects on vegetative growth (dwarfing) and increase of yield per canopy volume induced by HSVd and CDVd. Stuchi et al. (2007) related that 'Marsh Seedless' grapefruit grafted on 'Trifoliate' orange trees, when separately inoculated with two viroid combinations (CEVd + HSVd + CDVd and HSVd + CDVd), showed fruit quality similar to healthy ones, but the strong effect on tree size (84% and 62% less canopy volume for the combinations with and without CEVd, respectively) resulted in low yield.

In conclusion, the differences in the severity of "Quebra-galho" symptoms among different trees is probably associated with the presence (or absence) of CEVd, with its interaction with other viroids perhaps determining the different phenotypes observed in the field. In agreement with our results, Murcia et al. (2007) detected only CEVd or CEVd plus CDVd in three trees of acid lime 'Tahiti' presenting severe bark-cracking ("Quebra-galho"), while in a survey analyzing 80 'Tahiti' trees, Silva (2007) found

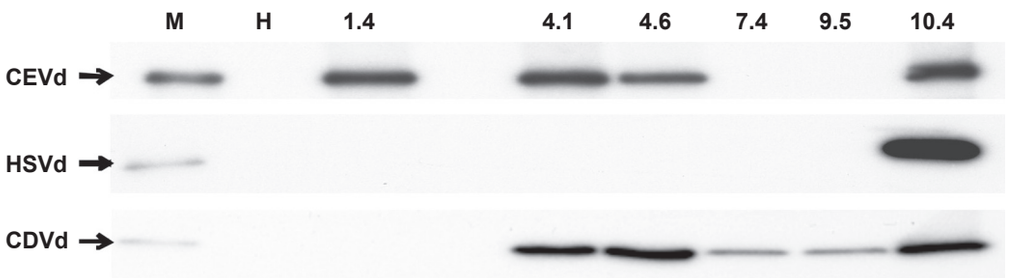

FIGURE 2 - Analysis by PAGE and Northern-blot hybridization with full-length complementary radioactively-labeled riboprobes of nucleic acid preparation from 'Etrog' citrons graft-inoculated with field viroid isolates from the acid lime 'Tahiti' clone "Quebra-galho" (1.4, 4.1, 4.6, 7.4, 9.5 and 10.4); H = Healthy control; M = Positive control.

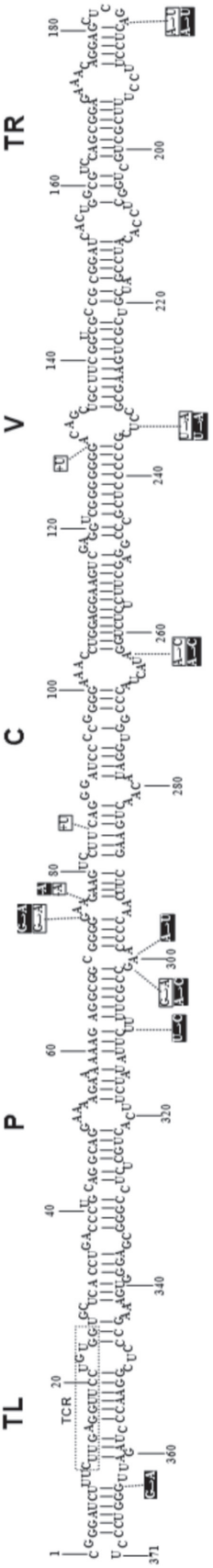

FIGURE 3 - Predicted secondary structure of minimum free energy for the plus strand of *Citrus exocortis viroid* variant CEVd-C (accession code J02053) with changes found in the two CEVd variants sequenced in this work: sample 4.6 (372-nt in length) and 10.4 (371-nt in length). Changes (residue substitutions) between these clones are indicated in the boxes: white and black boxes show the differences found in clones 4.6 and 10.4, respectively. The relative positions of the domains Terminal Right (TR), Variable (V), Central (C), Pathogenic (P) and Terminal Left (TL), as well as the Terminal Conserved Region (TCR), are indicated.

CEVd, HSVd and CDVd in different combinations, with all plants being at least infected by CEVd and approximately half by a mixture of CEVd and CDVd. Further assays are needed to establish the individual role of each viroid, or their combination, on the induction of bark-cracking symptoms characteristic of "Quebra-galho".

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support given by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Proc. 474522/2007-5). M. Eiras was supported by a Fundación Carolina (Madrid, Spain) research fellowship. Work in J.A.D. laboratory was supported by grants AGL2007-65653-C02-01 from Ministerio de Educación y Ciencia (Spain) and BIO2008-01986 from Ministerio de Ciencia e Innovación (MICINN, Spain), and work in R.F. laboratory by grants BFU2008-03154/BMC from MICINN and ACOMP/2010/278 from Generalitat Valenciana (Spain). We also thank Drs. N. Durán-Vila and P. Serra (IVIA, Moncada, Valencia, Spain) for kindly providing healthy citrus plants, citrus viroid markers and recombinant plasmids, and Dr. N. Durán-Vila for critical reading of the manuscript and excellent suggestions.

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TPP 123 - Received 25 April 2010 - Accepted 20 September 2010
Section Editor: F. Murilo Zerbini