Viroids in Tahiti Lime Scions Showing Bark Cracking Symptoms

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ABSTRACT. The so-called "quebra-galho" clone of Tahiti lime is very popular in Brazil, because the small size of the trees is suitable for high-density plantings. These Tahiti lime trees are easily recognized because they show bark-cracking symptoms, that have been claimed to be associated with "exocortis" infection. Viroid infection of three "quebra-galho" Tahiti lime trees from a farm near Araraquara in the state of São Paulo, Brazil, was assessed by Northern-blot hybridization using viroid-specific probes. Similarly, eight clones of Tahiti lime from different origins and available at Topara Nursery, near Chincha, Peru, were also tested for viroids. The four clones that displayed characteristic bark-cracking symptoms were found to be infected with *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus viroid III* (CVd-III), whereas the clones that did not show bark-cracking symptoms were either viroid-free or infected only with CVd-III. A study is being conducted to establish if viroids, and in particular CEVd, are indeed the cause of "quebra-galho" bark cracking symptoms. Preliminary observations indicated that 2-yr-old CEVd-infected lime trees at Moncada, Spain showed cracks.

Index words: Citrus viroids, CEVd, Quebra-galho, Wood pocket

In Brazil, the term "Quebra-galho" (branch breaker) refers to the main characteristic of certain Tahiti lime clones known as "Quebra-galho clones". In spite of their relatively short longevity, the trees show desirable traits such as small size suitable for high density planting, easy management (harvesting, phytosanitary treatments, etc.), multiple flowering, and production periods during which fruits from other cultivars are not available. Because of their characteristics, these clones are very popular among small growers in spite of physiological and phytosanitary problems that limit their use on certain rootstocks. It is generally accepted that the properties of "Quebra-galho clones" result from viroid infection. This is supported by the fact that "Quebra-galho clones" grafted on Rangpur lime develop bark scaling symptoms characteristic of exocortis. However, the cause and effect relationship between viroid infection and bark cracking of "Quebra-galho clones" has not been fully demonstrated. In the present study, we report that Tahiti lime trees showing bark cracking symptoms are infected with viroids, including the exocortis viroid. Preliminary results of a study being conducted to fulfill Koch's postulates are also presented.

MATERIALS AND METHODS

Plant materials. Samples were collected in July 2006 from three Tahiti lime trees growing on a farm near Araraquara in the state of São Paulo, Brazil, displaying characteristic bark cracking symptoms (Table 1, samples 1, 2, and 3).

TABLE 1
IDENTIFICATION OF CITRUS VIROIDS IN TAHITI LIME

Source	Bark cracking	Citrus viroids[a]					
		CEVd	HSVd	CBLVd	CVd-III	CVd-IV	CVd-V
(1) Brazil	+	+	-	-	+	-	-
(2) Brazil	+	+	-	-	-	-	-
(3) Brazil	+	+	-	-	+	-	-
(4) Mexico	-	-	-	-	-	-	-
(5) Peru	+	+	+	-	+	-	-
(6) Mexico	-	-	-	-	-	-	-
(7) Peru	+	+	+	-	+	-	-
(8) Peru	+	+	+	-	+	-	-
(9) Peru	-	-	-	-	+	-	-
(10) Peru	-	-	-	-	-	-	-
(11) Peru	+	+	+	-	+	-	-

[a]Viroids were detected by Northern blot hybridization using viroid-specific probes. Positive samples were confirmed by RT-PCR using viroid-specific primers.

Eight additional samples were collected in July 2006 from symptomatic (Table 1, samples 5, 7, 8, and 11) and symptomless (Table 1, samples 4, 6, 9, and 10) Tahiti lime trees in the Topara nursery located near Chincha, Peru. Samples 4 and 6 came from trees of Mexican origin. These samples were inoculated in graft-propagated Etrog citron plants in the greenhouse facilities at Bordeaux and analyzed 9 mo later.

Viroid analysis. Citron samples of young stems and leaves (5 g) were powdered in liquid nitrogen and homogenized in 5 ml of extraction medium (0.4 M Tris-HCl pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7.0; 4% (v/v) β-mercaptoethanol) and 15 ml of water-saturated phenol (19). The total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, pH 7.4; 10 mM KCl; 0.1 mM $MgCl_2$). These preparations were used for Northern blot hybridization and RT-PCR analysis.

For sequential polyacrylamide gel electrophoresis (sPAGE) analysis, 20-µl aliquots (equivalent to 300 µg fresh weight) were first subjected to a 5% non-denaturing polyacrylamide gel electrophoresis (PAGE) at 60mA for 2.5 h. Next, a segment of the ethidium bromide-stained gel containing CEVd and 7S RNA was subjected to a second PAGE containing 8M urea, at 18mA for 4 h (16). Viroid bands were visualized by silver staining (7).

For Northern blot hybridization, 20-µl aliquots (equivalent to 300 mg fresh weight) were subjected to 5% non denaturing PAGE and stained with ethidium bromide. The RNAs separated by 5% PAGE were electroblotted (400 mA for 2 h) to positively charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA), immobilized by UV cross-linking and hybridized with viroid-specific probes.

Digoxigenin (DIG)-labeled DNA probes were synthesized by PCR using as a template a cloned plasmid containing full-length viroid monomeric DNA, as described by Palacio-Bielsa, et al. (12) for *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV). A probe specific for the newly described *Citrus viroid V* (CVd-V) (20) was synthesized using primers CVd-V-h (5'-TCGACGAAGGCCGGTGAGCA-3') and CVd-V-c (5'-CGACGACAGGTGA GTACTCTCTAC-3'), respectively, that were homologous and complementary to positions 88-107 and 64-87 of the viroid-reference sequence. Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and 5xSSC buffer containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking reagent. After hybridization, the membranes were washed twice in 2xSSC, 0.1% SDS at room temperature for 15 min, and once in 0.1xSSC, 0.1% SDS at 60ºC for 60 min, and revealed with an anti-DIG alkaline phosphatase conjugate and the chemiluminiscence substrate CSPD (Roche Applied Science) (DIG-labeled probes).

RT-PCR was performed as described by Bernad and Duran-Vila (2). First-strand cDNA synthesized at 60ºC using 27-mer primers specific for each viroid and Thermoscript reverse transcriptase (Invitrogen®). In order to recover full-length viroid DNA, second-strand synthesis and DNA amplification were performed by using a set of two contiguous 18-mer forward and reverse primers specific for each viroid in 50-µl reactions containing 1.0 mM $MgCl_2$, 0.12 mM dNTPs, 0.5 µM of each primer and 1 U of *Taq* DNA polymerase. PCR parameters consisted of a 5-min denaturation at 94ºC followed by 35 cycles of 94ºC (30 s), 60ºC (30 s), 72ºC (1 min) and finishing with a 5-min extension step at 72ºC. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA products that were sequenced. When the sequences contained indeterminations, the amplification product was ligated in the pGEM-T vector (Promega) and the recombinant plasmids were used to transform DH5α *E. coli* cells.

Sequencing and sequence analysis. Uncloned amplicons synthesized by RT-PCR and/or recombinant plasmids were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple sequence alignments were performed with Clustal W (21).

Propagation and viroid inoculation of Tahiti lime trees. Cleopatra mandarin seedlings were established in a field plot located at the Instituto Valenciano de Investigaciones Agrarias (IVIA), at Moncada, Spain in June 2005. Six weeks later, the seedlings were graft-inoculated with one of six viroid sources that had been maintained in Etrog citron (four plants per viroid treatment and four non-inoculated controls in a randomized block arrangement). The isolates chosen were CEVd (CEVd-117) (6), CBLVd (CVd-Ia-117) (4), HSVd (X-707 and CVd-IIa-117) (13), CVd-III (CVd-IIId) (4), and CVd-IV (CVd-IV-Ca) (5). CEVd-117 had been characterized as a severe strain (6) highly homologous to the CEVd sequences defined by Visvader and Symons (22, 23) as class A. HSVd isolates (X-707 and CVd-IIa-117) had been characterized as cachexia and non-cachexia-inducing variants respectively (13).

Buds from a mature, viroid-free Tahiti lime tree (Bearss/IVIA-124), available at the IVIA germplasm bank (www.ivia.es), were graft propagated on the Cleopatra mandarin seedlings on October 2005. Viroid infection of all inoculated trees was confirmed by sPAGE analysis in October 2007.

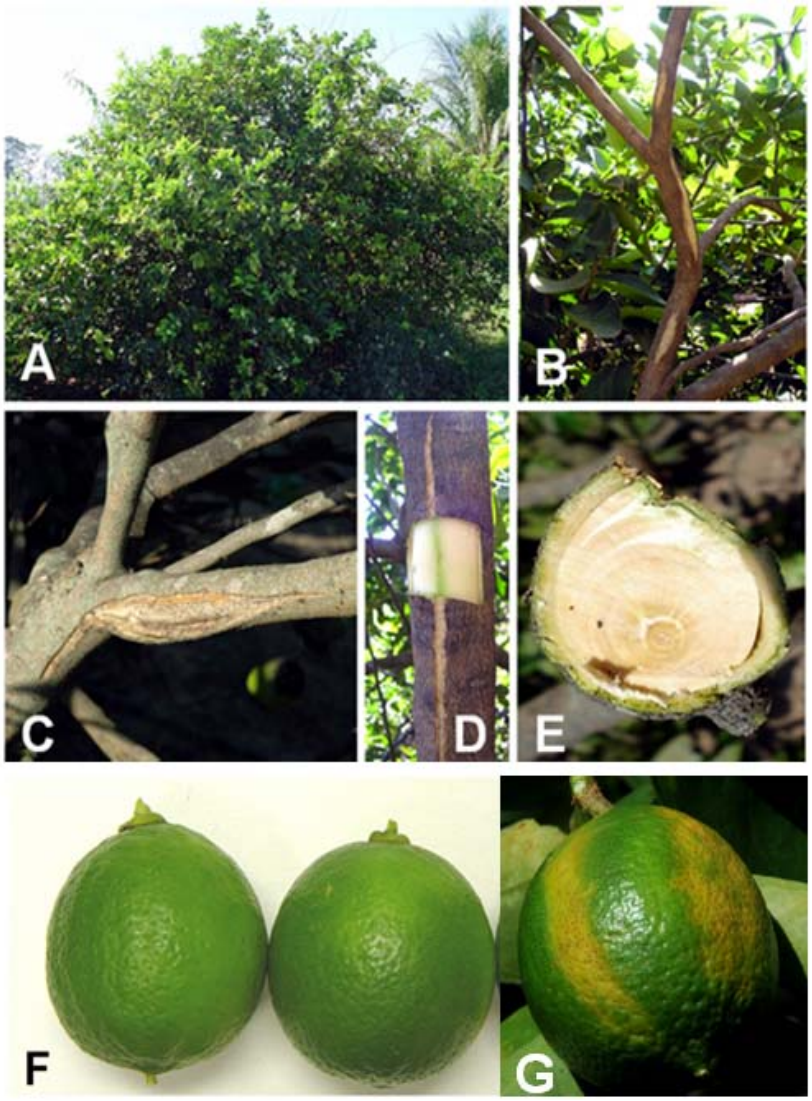

Fig. 1. Symptoms in Tahiti lime trees sampled in Brazil and Peru: A) Non-declining tree from Brazil; B-C) Bark cracking symptoms in trees sampled in Brazil and Peru; D-E) Lack of wood staining in trees sampled in Brazil and Peru; F) Lack of fruit sectoring symptoms; G) Fruit sectoring symptoms characteristic of the wood pocket syndrome.

RESULTS

Bark cracking symptoms in Tahiti lime. The symptomatic Tahiti lime trees sampled in Brazil and Peru did not show decline symptoms (Fig. 1A). They showed bark cracking symptoms characteristic of "Quebra-galho" clones (Fig. 1B, 1C), but were devoid of the wood staining (Fig. 1D, 1E) and the fruit sectoring (Fig. 1F, IG) associated with the "wood pocket syndrome" (8).

Identification of viroids in Tahiti lime trees showing bark cracking symptoms. Northern blot hybridization analysis of nucleic acid preparations from Etrog citron plants that had been graft

inoculated with the 11 Tahiti lime sources revealed the presence of CEVd, HSVd and CVd-III (Fig. 2). These results from the positive samples were confirmed by RT-PCR using viroid-specific primers. As summarized in Table 1, all three symptomatic clones from Brazil were infected with CEVd, two of the clones (samples 1 and 3) contained CVd-III in addition. Similarly, CEVd was present in all four symptomatic clones collected in Peru (samples 5, 7, 8, and 11), but these clones contained also HSVd and CVd-III. Three of the symptomless clones collected in Peru (samples 4, 6 originally from Mexico) and (sample 10 originally from Peru) were free of viroids, whereas only one (sample 9) contained CVd-III.

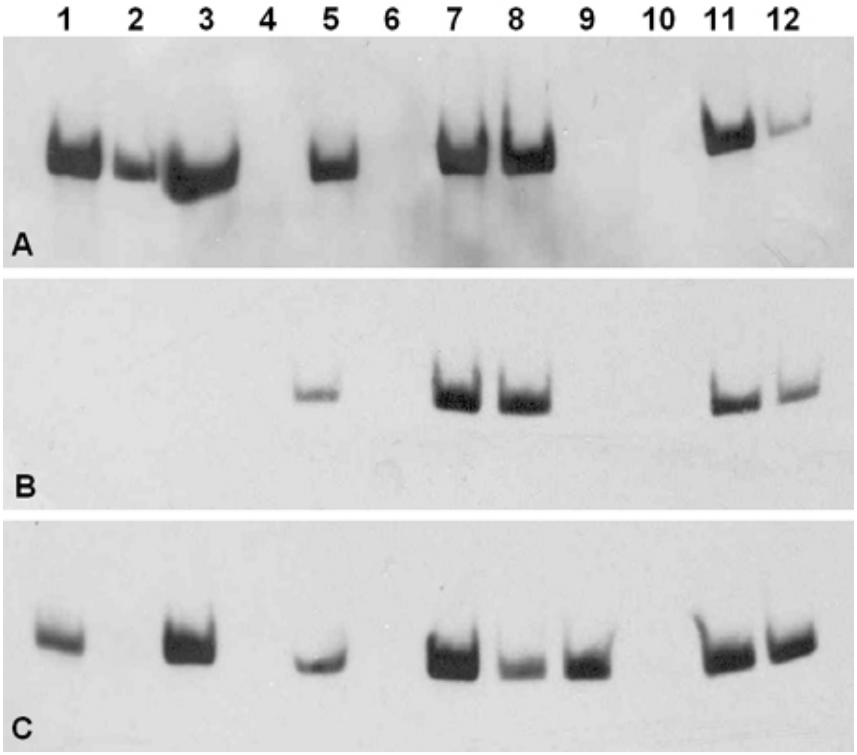

Fig. 2. Positive Northern blot hybridization analyses of Tahiti lime trees using specific probes for CEVd (A), HSVd (B) and CVd-III (C). Samples from Brazil: 1 to 3; samples collected in Peru: 4 to 11, including two samples of Mexican origin (samples 4 and 6); positive viroid control: 12.

Molecular characterization of viroids isolated from Tahiti lime. Sequence analysis of the uncloned RT-PCR amplicons obtained by using CEVd - specific primers showed that the three samples from Brazil contained very closely related CEVd sequences, with nucleotide identities ranging from 97.0 to 98.1% with the reference sequence of Class A, as defined by Visvader and Symons (22, 23). The four samples from Peru also contained virtually identical CEVd sequences, with sequence identities of 99.7% with the reference sequence of Class B, as also defined by Visvader and Symons (22, 23). These results indicated that the CEVd-infected Tahiti lime clones from Brazil (clones of samples 1, 2, and 3) and Peru

(clones of samples 5, 7, 8, and 11) had different origins.

Sequence analysis of the uncloned RT-PCR amplicons obtained using CVd-III specific primers showed that the two CVd-III-infected samples from Brazil (samples 1 and 3) contained closely related CVd-III sequences, with nucleotide identities ranging from 98.0 to 98.8% with the reference sequence of CVd-IIIa as defined by Rakowski et al. (14). The five CVd-III-infected samples from Peru (samples 5, 7, 8, 9, and 11) contained very closely related CVd-III sequences, with nucleotide identities ranging from 99.3 to 100% with the reference sequence of CVd-IIIb (Table 2), as also defined by Rakowski et al. (14). These results confirmed that the CVd-III infected Tahiti lime clones from Brazil and Peru had different origins.

TABLE 2
SEQUENCE IDENTITIES OF VIROIDS IDENTIFIED IN TAHITI LIME WITH THE TYPE MEMBERS OF CEVd, HSVd and CVd-III

Source	Sequence identities						
	CEVd[a]		HSVd[b]		CVd-III[c]		
	CEVd-A (M30868)	CEVd-B (M30870)	CVd-IIa (AF213503)	CVd-IIb (AF213501)	CVd-IIIa (S76452)	CVd-IIIb (AF184147)	CVd-IIIc (AF184149)
(1) Brazil	97.0	92.5	-	-	98.8	95.5	93.5
(2) Brazil	98.1	93.2	-	-	-	-	-
(3) Brazil	98.0	93.4	-	-	98.0	96.0	93.4
(5) Peru	93.5	99.7	99.3	97.0	96.0	99.3	94.5
(7) Peru	93.8	99.7	99.3	97.0	96.3	99.6	94.8
(8) Peru	93.8	99.7	99.3	97.0	96.2	99.6	94.8

Sequence analysis of the uncloned RT-PCR amplicons obtained using HSVd specific primers showed that the four HSVd-infected samples from Peru (samples 5, 7, 8, and 11) had nucleotide identities of 99.3% with the reference sequence of CVd-IIa, as defined by Reanwarakorn and Semancik (15) and biologically characterized as a non-cachexia strain of HSVd.

Symptom expression of Tahiti limes inoculated with citrus viroids. Buds from a mature, viroid-free and symptomless Tahiti lime tree in the germplasm collection of IVIA (Fig. 3A) were graft propagated on Cleopatra mandarin rootstock seedlings experimentally infected with CEVd, HSVd, CBLVd, CVd-III or CVd-IV. After 2 yr, the Tahiti lime scions of the trees showed small cracks in the branches (Fig. 3B) that were also present in some of the non-inoculated controls. The average number of cracks per tree ranged from 3.5 (non-inoculated control) to 4.0 (non-cachexia HSVd), 4.5 (CBLVd and CVd-IV), 9.0 (cachexia HSVd), 10.5 (CVd-III) and 76.3 (CEVd). One-way analysis of variance showed that only CEVd-infected trees had significantly more cracks than those of all the other treatments and the non-inoculated control ($P<0.05$).

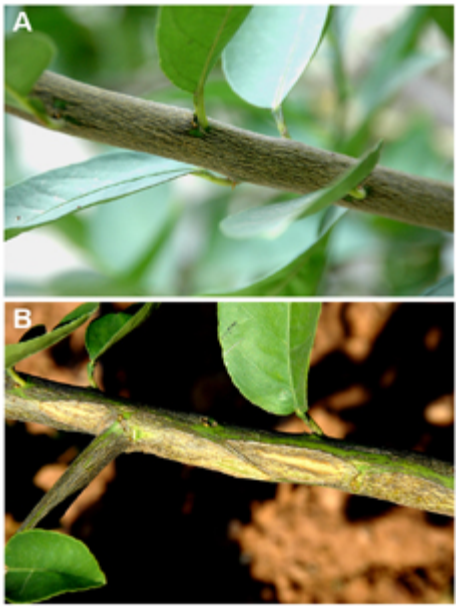

Fig. 3. A) Viroid-free, symptomless, mature Tahiti lime tree devoid of bark cracks used for graft propagation of lime buds on CEVd-infected Cleopatra mandarin rootstock seedlings. B) Bark cracking symptoms observed on the CEVd infected Tahiti lime trees on Cleopatra mandarin growing in the field since 2005.

DISCUSSION

Assays to determine the cause of bark cracking of Tahiti lime were initiated in 1961 by Salibe and Moreira (18) who indexed several Tahiti lime selections with bark cracking symptoms on Rangpur lime, an exocortis-indicator used at that time. The severity of the exocortis "strains" in the various Tahiti lime selections was judged in particular by the size of the yellow areas and extent of cracking in the bark of the Rangpur lime sprouts. They found a significant correlation between the presence of bark cracking on the Tahiti lime trees and the development of exocortis symptoms on the inoculated Rangpur lime seedlings. Furthermore, they were able to reproduce the bark cracking symptoms by inoculating a Tahiti lime source of presumed nucellar origin with several "exocortis" sources (18). This early work, carried out at a time when viroids had not yet been identified, clearly demonstrated that: (i) a graft-transmissible agent, supposedly a virus, was involved as the causal agent of the bark cracking syndrome observed in Brazil; (ii) the bark cracking symptoms observed in Brazil were unrelated to a similar disorder of Tahiti lime first described by Ruehle (17) and characterized by leaf blotching, fruit sectoring, breaks in the bark of the trunk, and wood staining. This disorder was later reported as "lime blotch", a disorder similar to "wood pocket" of lemon (3, 8), and it is presently considered to be a physiological or genetic disorder of certain Tahiti lime clones.

The work of Salibe and Moreira (18) was conducted, as indicated above, before the causal agent of the exocortis disease was known to be a viroid and, therefore, their classification of exocortis "strains" as severe, moderate, or mild, needed to be re-considered on the basis of the two following facts that: (i) CEVd sources may also carry other citrus viroids and (ii) some mild "exocortis" sources are now known to be caused by viroids other than CEVd. Various reports show that Tahiti lime trees showing bark cracking symptoms are indeed infected with several viroids (1, 10, 11), but the cause and effect relationship had not been demonstrated in these reports.

The results of the present study support and extend the work of Salibe and Moreira (18) and show that. (i) "Quebra-galho" is different from lime blotch or lemon wood pocket, (ii) viroid analysis of different Tahiti lime sources shows a direct correlation only with CEVd infection, and (iii) young Tahiti lime trees inoculated with CEVd developed significant numbers of small bark cracks within two years. The further development of some bark cracks observed even in the non-inoculated trees should be adequately assessed. Complete fulfillment of Koch's postulates will require

more time for these symptomatic lime trees for the development of the small cracks into characteristic symptoms of "Quebra-galho". Additional surveys of more bark cracking-affected Tahiti lime trees would provide additional information regarding whether or not viroids other that CEVd can cause this bark cracking symptoms.

ADDENDUM

Since this information was presented at the 17th IOCV Conference held in 2007, the International Committee on Taxonomy of Viruses (ICTV) has accepted some changes in the viroid nomenclature. The new names for CVd-III and CVd-IV are *Citrus dwarfing viroid* and *Citrus bark cracking viroid*, respectively.

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