Resumen

Los cítricos son huéspedes naturales de diferentes especies de viroides, todos pertenecientes a la familia *Pospiviroidae*. Estudios preliminares para detectar viroides en especies y variedades comerciales dieron resultados erráticos, excepto cuando se utilizaba la especie indicadora cidro Etrog como huésped bioamplificador. Para evitar el uso de este huésped en los ensayos de detección, se desarrolló un método basado en la hibridación northern. El protocolo desarrollado consistía en: (i) extracción de ácidos nucleicos de muestras de corteza recolectadas en diferentes épocas y/o almacenadas en distintas condiciones; (ii) separación de los RNAs en 5% PAGE o 1% agarosa y transferencia a membranas; (iii) hibridación con sondas de DNA marcadas con digoxigenina (DIG) específicas para cada viroide, detección con un anticuerpo anti-DIG conjugado con fosfatasa alcalina y revelado con un substrato quimioluminiscente (CSPD). Con este método se pueden detectar viroides en todas las especies de cítricos ensayadas. La técnica es extremadamente sensible, y acorta el tiempo necesario para la detección fiable de los viroides de cítricos conocidos hasta el momento.

La aplicación de esta técnica ha permitido realizar prospecciones en árboles cultivados en distintas regiones citrícolas de Colombia, Perú y Brasil. En limas Tahití de Colombia se han identificado infecciones múltiples con HSVd y CDVd o con CEVd, HSVd y CDVd. Las muestras procedentes del Banco de Germoplasma de Palmira estaban libres de viroides, excepto una fuente de cidro Etrog que estaba infectada con CEVd y CDVd. La caracterización molecular de los aislados de HSVd, CDVd y CEVd recuperados de las muestras analizadas ha mostrado que: (i) todos los aislados de HSVd carecían del motivo de expresión de la caquexia, por lo que se trata de variantes no patogénicas, excepto uno que si contenía dicho dominio; (ii) los aislados de CDVd contenían variantes con alta identidad nucleotídica con la variante de referencia CVd-IIIb; (iii) uno de los aislados de CEVd (CEVd^{CO}) procedente de un cidro Etrog asintomático presentaba una identidad nucleotídica del 96,5% con la secuencia de referencia de la clase A (que engloba las variantes agresivas de este viroide) y del 98,9% con la secuencia de referencia V1 que induce síntomas muy agresivos en cidro Etrog. Los cambios nucleotídicos entre CEVd^{CO} y la variante V1 son: U186A (dominio T_R), A235U (dominio V) y AU313-314GA (dominio P). Dada la probada implicación del dominio P en la patogénesis del CEVd, se han obtenido mutantes en las posiciones 313-314 de V1 y CEVd^{CO} para poder demostrar si los cambios identificados en CEVd^{CO} son efectivamente los responsables de que los cidros infectados con dicho aislado de CEVd no manifiesten síntomas.

El análisis de clones de lima Tahití procedentes de Perú y Brasil que manifiestan el síndrome del "quebra-galho" (expresión que en portugués significa "rama quebradiza"), caracterizado por el agrietado de la corteza, enanismo y porte abierto de la copa, pero con características productivas deseables, ha mostrado que se encontraban infectados con CEVd, HSVd y CDVd (clones de Perú) o con CEVd y CDVd (clones de Brasil). Los resultados de un estudio realizado en condiciones de campo para determinar si estos viroides son los responsables del síndrome del "quebra-galho" han mostrado que el CEVd induce agrietado de la corteza en lima Tahití después de dos años de efectuada la inoculación, pero se requiere un mayor desarrollo de los árboles, para confirmar que este viroide es efectivamente responsable todos los síntomas característicos del síndrome del "quebra-galho".

El viroide del enanismo de los cítricos (CDVd) se encuentra muy difundido en diferentes regiones citrícolas pero sus efectos pasan inadvertidos debido a que no produce síntomas específicos a pesar de haberse probado que causa una reducción del tamaño del árbol. Se ha realizado un estudio exhaustivo dirigido a identificar y caracterizar distintas variantes de este viroide a partir de 33 aislados. El análisis SSCP ha mostrado que cada aislado contenía poblaciones de variantes de secuencia, y con la información obtenida se identificó y secuenció la variante más frecuente, y por tanto la más representativa de cada aislado. El análisis filogenético ha permitido establecer su relación con las variantes de referencia (CVd-IIIa, CVd-IIIb y CVd-IIIc). Se seleccionaron cinco secuencias representativas de cada uno de los cinco grupos mayoritarios del árbol filogenético para su caracterización biológica en cidro Etrog. Con los datos de crecimiento y la expresión de síntomas se ha establecido que dos variantes se comportaron como razas agresivas, dos como moderadas y una como muy suave. Los cambios encontrados en estas variantes fueron relacionados con la modulación de los síntomas.

Para analizar el efecto de los viroides en el comportamiento del cultivo en campo, se realizó el seguimiento y toma de datos de una parcela establecida con árboles de naranjo dulce Washington navel injertado en citrange Carrizo e inoculados con doce fuentes de viroides. El análisis estadístico de los parámetros estudiados (altura del árbol, grosor del tallo, tamaño de la copa, y cantidad y calidad de la cosecha) tomados durante cuatro campañas sucesivas mostraron que existían pocas diferencias estadísticas entre los distintos tratamientos.

Abstract

Citrus are natural hosts of several viroid species, all of them belonging to the family *Pospiviroidae*. Preliminary approaches to detect viroids in commercial species and cultivars yielded erratic results unless the indicator species Etrog citron was used as a bioamplification host. To avoid the use of this host in routine detection assays, a northern hybridization protocol was developed. This protocol consisted of: (i) Nucleic acid extraction of bark samples collected in different growing seasons and/or stored at different conditions; (ii) separation of the RNAs in 5% PAGE or 1% agarose and transfer to membranes; (iii) hybridization with viroid specific DNA probes labelled with digoxigenin (DIG), detection with an anti-DIG antiobody conjugated with alkaline phosphatase and developed with a chemiluminiscence substrate (CSPD). With this approach, viroids were successfully detected in all the citrus species and cultivars tested. The technique is extremely sensitive, and all the viroids described in citrus can be detected in a short period of time.

This technique was used to perform viroid surveys in different citrus growing areas of Colombia, Peru and Brazil. Multiple viroid infections of HSVd and CDVd or CEVd, HSVd and CDVd were found in Tahiti lime trees from Colombia. With the exception of a single Etrog citron tree that was found to be infected with CEVd and HSVd, the samples collected in the Germplasm Bank located in Palmira were viroid-free. The molecular characterization of the field isolates of HSVd, CDVd and CEVd recovered from the surveyed samples showed that: (i) With a single exception, all the HSVd isolates were devoid of the cachexia expression motif and therefore can be considered as non-pathogenic variants; (ii) the CDVd isolates contained variants with high sequence identity with the reference sequence CVd-IIIb; (iii) one of the CEVd isolates (CEVd^{CO}) recovered from a symptomless Etrog citron presented 96.5% nucleotide identity with the reference sequence of Class A (which contains the severe variants of this viroid) and 98.9% identity with the reference sequence V1 that induces severe symptoms in Etrog citron. The nucleotide differences between CEVd^{CO} and variant V1 are: U186A (T$_R$ domain), A235U (V domain) and AU313-314GA (P domain). Since the P domain is associated with the pathogenesis of CEVd, synthetic mutants in the nucleotides 313-314 of V1 and CEVd^{CO} were obtained with the aim to demonstrated if the changes identified in CEVd^{CO} are indeed responsible of the lack of symptom expression in the Etrog citron trees infected with this variant.

The analysis of several Tahiti lime clones from Peru and Brazil showing the "quebra-galho" syndrome (portuguesse expression meaning "breaking branche") and presenting the characteristic bark cracking, dwarfing and open canopy associated with other desirable traits,

showed that they were infected with CEVd, HSVd and CDVd (clones from de Peru) or with CEVd and CDVd (clones from Brazil). The results available from a field assay designed to find out if these viroids were responsible form the "quebra-galho" syndrome showed that CEVd caused bark cracking in Tahití lime trees two after inoculation. Additional observations in fully developed trees are required to confirm that CEVd is the causal agent of the overall symptoms characteristic of the "quebra-galho" syndrome.

Citrus dwarfing viroid (CDVd) is very widespread in different citrus growing areas but since it does not induce specific symptoms, its effect on tree size is poorly recorded. A study was addressed to identify and characterize variants of this viroid in 33 field isolates. SSCP analysis showed that each isolate contained populations of sequence variants and the most frequent variant of each isolate was identified and sequenced. A phylogenetic analysis demonstrated their relationship with the reference variants (CVd-IIIa, CVd-IIIb y CVd-IIIc). Five sequences representing the five major groups identified in the phylogenetic tree were selected for their further biological characterization in Etrog citron. Statistical analysis of growth parameters and symptom expression data showed that two variants acted as severe strains, two as moderate strains and single variant was a very mild strain. The nucleotide differences found in these variants were associated with the modulation of symptom expression.

In order to determine the effect of viroid infection on the performance of field grown trees, a field plot established with viroid-infected Washington navel sweet orange trees grafted in Carrizo citrange was monitored over four growing seasons. Statistical analysis of growth parameters (tree height, stem width, canopy volume and quantity and quality of fruit crop) showed only minor slight statistical differences among treatments.

Resum

Els cítrics son hostes naturals de varies espècies de viroides, totes pertanyent a la família *Pospiviroidae*. Estudis preliminars per a detectar viroides en espècies i varietats comercials donaren uns resultats molt erràtics, a no ser que s'utilitzés l'espècie indicadora cidre Etrog com a hoste bioamplificador. Per tal d'evitar la utilització d'aquest hoste en els assaigs de detecció rutinària de viroides es desenvolupà un mètode basat en la hibridació "northern". El protocol consistí en: (i) Extracció d'àcids nuclèics de mostres d'escorça preses en diferents èpoques de l'any i/o emmagatzemades en diferents condicions; (ii) separació dels RNAs en 5% PAGE o en 1% agarosa i transferència a membranes; (iii) hibridació amb sondes de DNA específiques per cada viroide i marcades amb digoxigenina (DIG), detecció amb un anticòs anti-DIG conjugat amb fosfatasa alcalina y revelat amb un substrat quimioluminiscent (CSPD). Amb aquest mètode s'han pogut detectar els viroides en totes les espècies de cítrics assajades. La tècnica es molt sensible, i permet reduir el temps necessari per a una detecció fiable de tots els viroides de cítrics descrits fins ara.

Aplicant eixa tècnica s'han pogut realitzar prospeccions de viroides en arbres cultivats en varies regions citrícoles de Colombia, Perú i Brasil. En llimes Tahití de Colombia s'hi han identificat infeccions múltiples de HSVd i CDVd o de CEVd, HSVd i CDVd. Les mostres del Banc de Germoplasma de Palmira estaven exemptes de viroides, excepte una font de cidre Etrog que estava infectada amb CEVd i CDVd. La caracterització molecular dels aïllaments de HSVd, CDVd i CEVd recuperats de les mostres analitzades ha mostrat que: (i) tots els aïllaments de HSVd no tenien el motiu d'expressió de la caquexia, i per tant es tracta de variants no patogèniques, excepte un que si que tenia eixe domini; (ii) els aïllaments de CDVd tenien variants amb una elevada identitat nucleotídica respecte a la variant de referència CVd-IIIb; (iii) un dels aïllaments de CEVd (CEVd^{CO}) procedent d'un cidre Etrog asintomàtic presentava una identitat nucleotídica del 96,5% amb la seqüència de referència de la classe A (que engloba les variants agressives d'eixe viroide) i del 98,9% amb la seqüència de referència V1 que causa símptomes molt agressius en cidre Etrog. Els canvis nucleotídics entre CEVd^{CO} i la variant V1 son: U186A (domini T_R), A235U (domini V) i AU313-314GA (domini P). Donada la comprovada implicació del domini P en la patogènesi del CEVd, s'han obtingut mutants artificials en las posicions 313-314 del V1 i del CEVd^{CO} per tal de poder demostrar si els canvis identificats en el CEVd^{CO} eren efectivament els responsables de que els cidres infectats amb aquesta font de CEVd no mostressin símptomes.

L'anàlisi d'una sèrie de clons de llima Tahití procedents del Perú i del Brasil i que manifestaven el síndrome del "quebra-galho" (expressió que en portuguès significa "rama trencadissa"), i que està caracteritzat per la formació d'esquerdes a l'escorça, nanisme i copa molt oberta, però que per altre banda tenen característiques productives interessants, ha mostrat que estaven infectats amb CEVd, HSVd i CDVd (clons del Perú) o amb CEVd i CDVd (clons del Brasil). Els resultats d'un estudi realitzat en condiciones de camp per tal de determinar si eixos viroides eren els responsables del síndrome del "quebra-galho" han mostrat que el CEVd causa esquerdes a l'escorça dels arbres de llima Tahití desprès de dos anys d'haver estat inoculats, però es necessari un major desenvolupament dels arbres, per poder confirmar que eixe viroide es efectivament responsable tots els símptomes característics del síndrome del "quebra-galho".

El viroide del nanisme dels cítrics (CDVd) està molt dispers en les regions citrícoles del mon però els seus efectes passen inadvertits ja que no indueix símptomes específics malgrat haver-se provat que causa una reducció de la mida dels arbres infectats. Partint de 33 aïllaments s'ha realitzat un estudi per tal d'identificar i caracteritzar variants d'eixe viroide. L'anàlisi per SSCP ha mostrat que cada aïllat estava compost per poblacions de variants de seqüència, i amb la informació obtinguda s'ha identificat y seqüenciat la variant mes freqüent, que es per tant la mes representativa de cada cas. Amb els resultats de l'anàlisi filogenètic s'ha determinat la seua relació amb les variants de referència (CVd-IIIa, CVd-IIIb i CVd-IIIc). S'han seleccionat cinc seqüencies representatives de cada un dels cinc grups majoritaris de l'arbre filogenétic per tal de caracteritzar-los biològicament en l'espècie indicadora cidre Etrog. Amb l'anàlisi de les dades de creixement i d'expressió de símptomes s'ha vist que dues variants es comportaven com a races agressives, dues com a moderades y una com a molt suau. Els canvis típics d'eixes variants s'han relacionat amb la modulació dels símptomes.

Per tal d'analitzar l'efecte dels viroides en el comportament del cultiu en el camp, es realitzà un seguiment i presa de dades dels arbres d'una parcel·la de taronger Washington navel empeltat en citrange Carrizo i inoculats amb dotze fonts de viroides. L'anàlisi estadístic dels paràmetres estudiats (alçada del arbre, grossor del tronc, mida de la copa, i quantitat i qualitat de la collita) presos durant quatre campanyes successives han mostrat que hi han molt poques diferencies estadístiques entre els tractaments.

ÍNDICE

Introducción

1. Aspectos Generales

Los viroides son los agentes infecciosos subvirales más sencillos genética y estructuralmente descritos en la naturaleza. Están conformados por una molécula de RNA circular de cadena simple con un tamaño que varía entre 246 y 401 nucleótidos. Los viroides son mucho más simples que los virus ya que carecen de envoltura proteica y dependen totalmente de la maquinaria transcripcional del huésped para su replicación. Son una clase de RNAs que no codifican proteínas y que infectan y se replican únicamente en plantas superiores tanto monocotiledóneas como dicotiledóneas en las que pueden causar enfermedades (Sänger *et al*., 1976; Haseloff *et al*., 1982; Diener, 2003; Flores *et al*., 2005a; Ding e Itaya, 2007).

Los primeros estudios sobre estos agentes patógenos se iniciaron cuando se intentaba determinar el agente causal de la enfermedad del tubérculo fusiforme de la patata en la década de 1970. Aunque las tecnologías disponibles eran muy limitadas, los estudios realizados permitieron demostrar que se trataba de un agente infeccioso compuesto de un RNA de bajo peso molecular, no encapsidado y con replicación autónoma, características que permitieron diferenciarlo de los virus (Diener, 1971) e introducir el concepto de viroide. En los años siguientes a la descripción del primer viroide, el viroide del tubérculo fusiforme de la patata (*Potato spindle tuber,* PSTVd), se describieron otros dos viroides, el viroide de la exocortis de los cítricos (*Citrus exocortis viroid*, CEVd) que es el agente causal de la exocortis (Semancik y Weathers, 1972a) y el viroide del enanismo del crisantemo (*Chrysanthemum stunt viroid*, CSVd) (Diener y Lawson, 1972).

En la actualidad se han descrito más de 30 especies de viroides (Tabla 1) pero hasta el momento solo 29 han sido reconocidas como tales por el Comité Internacional de Taxonomía de Viruses (ICTV) (www.ictvonline.org/virusTaxonomy.asp), siendo PSTVd el primer viroide secuenciado (Gross *et al*., 1978). Según las normas establecidas por el ICTV y atendiendo a sus propiedades biológicas y moleculares, los viroides se encuentran agrupados en dos familias taxonómicas (Flores *et al.*, 2005a): *Pospiviroidae* cuya especie tipo es PSTVd (Diener, 1971; Gross *et al.,* 1978), y *Avsunviroidae* cuya especie tipo es el viroide del manchado solar del aguacate (*Avocado sunblotch viroid,* ASBVd) (Symons, 1981).

Tabla 1. Listado de todos los viroides descritos (Flores *et al.,* 2005).

Acrónimo	Nombre	Tamaño (nt)	Referencia
PSTVd	**Viroide del tubérculo fusiforme de la patata**	**356,359-360**	**Gross *et al.*, 1978**
TCDVd	Viroide del enanismo clorótico del tomate	360	Singh *et al.*, 1999
TPMVd	Viroide de la planta macho del tomate	360	Kiefer *et al.* 1983
MPVd	Viroide de la papita mexicana	359-360	Martínez-Soriano *et al.*, 1996
CEVd	Viroide de la exocortis de los cítricos	370-375	Gross *et al.,* 1992
CSVd	Viroide del enanismo del crisantemo	354,356	Haseloff y Symons, 1981
TASVd	Viroide del enanismo apical del tomate	360,363	Kiefer *et al.,* 1983
IrVd1	Viroide 1 de *Iresina*	370	Spieker, 1996a
CLVd	Viroide latente de *Columnea*	370,372-373	Hammond *et al.*,1989
PCFVd	Viroide del "Pepper chat" del pimiento[5]	348	Verhoeven *et al.*, 2009
HSVd	**Viroide del enanismo del lúpulo**	**297-303**	**Ohno *et al.*, 1983**
CCCVd	**Viroide del cadang-cadang del cocotero**	**246-247**	**Haseloff *et al.*, 1982**
CtiVd	Viroide del tinangaja del cocotero	254	Keese *et al.*, 1988
HLVd	Viroide latente del lúpulo	256	Puchta *et al.*, 1988a
CBCVd	Viroide de la corteza agrietada de los cítricos	284	Puchta *et al.*, 1991
ASSVd	**Viroide de la piel cicatrizada de la manzana**	**329-330**	**Hashimoto y Koganezawa, 1987**
CDVd	Viroide del enanismo de los cítricos	294-297	Rakowsky *et al.*, 1994
ADFVd	Viroide del fruto picado del manzano	306	Di Serio *et al.*, 1996
GYSVd-1	Viroide 1 del moteado amarillo de la vid	366-368	Koltunow y Rezaian, 1988
GYSVd-2	Viroide 2 del moteado amarillo de la vid	363	Koltunow *et al.*, 1989
CBLVd	Viroide de la hoja curvada de los cítricos	318	Ashulin *et al.*, 1991
PBCVd	Viroide de los chancros pustulosos del peral	315-316	Hernández *et al.*, 1992
AGVd	Viroide australiano de la vid	369	Rezaian, 1990
CVd-V	Viroide V de los cítricos[2]	294	Serra *et al.*, 2008
CVd-OS	Viroide OS[1]	330-331	Ito,*et al.*, 2001
PVd	Viroide del Caqui[3]	396	Nakaune *et al.*, 2008
CbVd-1	**Viroide 1 del coleus blumei**	**248,251**	**Spieker *et al.*, 1990**
CbVd-2	Viroide 2 del coleus blumei	301	Spieker, 1996b
CbVd-3	Viroide 3 del coleus blumei	361,362,364	Spieker *et al.*, 1966a
CbVd-5	Viroide 5 del coleus blumei[4]	274	Hou *et al.*, 2009
ASBVd	**Viroide del manchado solar del aguacate**	**246-250**	**Symons, 1981**
PLMVd	**Viroide del mosaico latente del melocotonero**	**335-338**	**Hernández y Flores, 1992**
CChMVd	Viroide del moteado clorótico del crisantemo	399	Navarro y Flores, 1997
ELVd	**Viroide latente de la berenjena**	**332-335**	**Fadda *et al.*, 2003**

[1,2,3,4,5] Viroides descritos en la literatura pero todavía no aceptados por el Comité Internacional de Taxonomía de Virus

2. Características Moleculares

2.1 Estructuras primaria

El análisis de la estructura primaria de los viroides, permitió agruparlos en dos familias *Pospivirodae* y *Avsunviroidae* (Tabla 2). Los viroides de la familia *Pospiviroidae* poseen un alto grado de complementariedad interna (entre el 64 y el 73% de bases apareadas) y un alto contenido en G+C (mas del 50% del total de nucleótidos) mientras que los de la familia *Avsunviroidae* presentan un contenido en G+C muy inferior, solo del 38% en el caso del ASBVd e intermedio (53-54%) en el caso del viroide latente de la berenjena (*Eggplant latent viroid*, ELVd) (Fadda *et al.*, 2003).

2.2 Estructura secundaria y dominios estructurales

Mediante programas informáticos y en base a cálculos termodinámicos se ha determinado la estructura secundaria de mínima energía libre que puede adoptar el RNA de los viroides y que por tanto se consideran como sus estructuras más estables (Zucker, 1989). En condiciones no desnaturalizantes, los viroides adoptan estructuras en forma de varilla, casi-varilla o ramificada en las que regiones de apareamiento elevado alternan con pequeños bucles formados por bases desapareadas (Riesner, 1987; Dingley *et al.*, 2003; Sänger *et al.*, 1976). Si bien para la mayor parte de los viroides la conformación más estable es la forma de varilla, hay algunas excepciones como ASBVd, viroide de los chancros pustulosos del peral (*Pear blister cancker viroid*, PBCVd) (Hernández *et al*., 1992), viroide del fruto picado del manzano (*Apple dimple fruit viroid*, ADFVd) (Di Serio *et al*., 1996) y ELVd (Fadda *et al.,* 2003a) que presentan estructuras de casi varilla, mientras que otros como el viroide del mosaico latente del melocotonero (*Peach latent mosaic viroid*, PLMVd) (Hernández y Flores, 1992) y el viroide del moteado clorótico del crisantemo (*Chrysanthemun chlorotic mottle viroid*, CChMV) presentan estructuras altamente ramificadas (Navarro y Flores, 1997).

Los viroides de la familia *Pospiviroidae* presentan en la zona central de la estructura secundaria de la molécula una región central conservada (**C**entral **C**onserved **R**egion, CCR) formada por dos segmentos de nucleótidos conservados, en la cual la hebra superior está flanqueada por repeticiones invertidas. Además de la CCR, los viroides pueden presentar otros motivos conservados, la región terminal conservada (**T**erminal **C**onserved **R**egion, TCR) y horquilla terminal conservada (**T**erminal **C**onserved **H**airpin, TCH). La TCR es un motivo de 13 a 16 nucleótidos situado en la hebra superior de la región terminal izquierda mientras que la TCH es un motivo de 13 nucleótidos localizados en el extremo terminal izquierdo. Dependiendo de la naturaleza de la CCR y de la presencia o ausencia de TCR o TCH, los miembros de esta familia se clasifican en cinco géneros (Koltunow y Rezaian, 1989). La TCR se encuentra presente en los viroides de los géneros *Apscaviroid*, *Pospiviroid* y en los dos miembros de mayor tamaño del género *Coleviroid* (CbVd-2 y CbVd-3) (Flores *et al.*, 1997), mientras que la TCH se encuentra en los miembros de los géneros *Hostuviroid* y *Cocadviroid* todo ellos con un tamaño menor o igual a 300 nucleótidos y sin TCR (Puchta *et al*., 1988; Flores *et al.*, 1997). La conservación de estas regiones tanto en su composición como en su ubicación sugiere que podrían tener un papel funcional importante que por el momento se desconoce.

Keese y Symons (1985) elaboraron un modelo estructural basado en las similitudes de secuencia observadas en los distintos viroides conocidos hasta aquel momento y en el que diferenciaron cinco dominios en la estructura de varilla. El dominio central (**C**entral**,** C) que se

halla flanqueado por los dominios patogénico (**P**athogenic, P) y variable (**V**ariable, V), y éstos por los dominios terminales derecho (**T**erminal **r**ight, T$_R$) e izquierdo (**T**erminal **l**eft, T$_L$) situados en los extremos de la estructura secundaria de la molécula (Figura 1). Este modelo se ajusta a los viroides de la familia *Pospiviroidae* (Flores *et al.,* 1999).
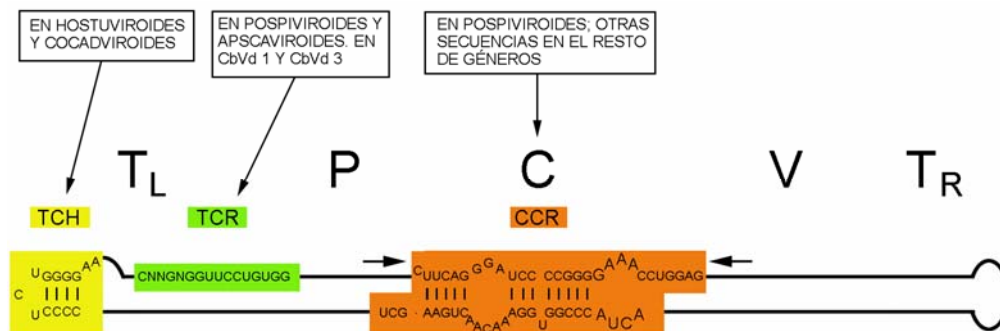
Fig. 1. Dominios estructurales de los viroides basado el modelo propuesto por Keese y Symons (1985) y adaptado de Flores *et al.* (1997). En colores destacan los motivos conservados para los viroides de la familia *Pospiviroidae*.

En base a los conocimientos que se tenían acerca de los viroides descritos en 1985 cuando se propuso este modelo, Keese y Symons (1985) trataron de asignar una función a cada dominio. El dominio P se relacionó con los efectos patogénicos del PSTVd y otros viroides estrechamente relacionados con éste, y el dominio C se asoció con la replicación. Hoy en dia se conoce que la interacción entre dominio y función es más compleja.

Dominio Central (C): Es la región más conservada e incluye la CCR que sirve de criterio para la taxonomía de los viroides (Koltunow y Rezaian, 1989).

Dominio Patogénico (P): Este dominio se relacionó con los efectos patogénicos de PSTVd (Gora *et al.,* 1996) y otros viroides estrechamente relacionados con éste, y se validó con experimentos realizados con quimeras intraespecíficas de CEVd (Visvader y Symons, 1986). Se intercambiaron los dominios P de aislados de distinta agresividad y se encontró una correlación entre la composición de este dominio y la expresión de síntomas. Sin embargo, en el caso de las cepas del viroide del enanismo del lúpulo (*Hop stunt viroid*, HSVd) que causan la enfermedad de la caquexia de los cítricos, se ha demostrado que los determinantes de la patogenicidad se hallan en otro dominio (Reanwarakorn y Semancik, 1998). Sin embargo, es probable que los mecanismos implicados en la inducción de síntomas sean más complejos de lo que inicialmente se había creído, y estén controlados por la interacción de determinantes

discretos situados en varios dominios (Sano *et al.*, 1992; Sano e Ishiguro, 1998; Reanwarakorn y Semancik, 1998, Qi y Ding, 2003).

Dominio Variable (V): Este dominio se presenta como altamente variable tanto entre secuencias de viroides relacionados como entre variantes de un mismo viroide. En los estudios realizados por Visvader y Symons (1986) con quimeras artificiales, se menciona que este dominio podría regular la acumulación del viroide en la planta huésped. Una excepción a este modelo se presenta en el caso de HSVd (único miembro del género *Hostuviroid*) en cítricos ya que es en este dominio donde se ubican los determinantes de la patogenicidad tal como se demostró en experimentos realizados con quimeras intraespecíficas entre variantes de diferente patogenicidad, así como con la introducción de cambios puntuales en el dominio V mediante mutagenésis dirigida (Reanwarakorn y Semancik, 1998; Serra *et al.,* 2008a).

Dominios Terminales (T_L y T_R): Se ha planteado que estos dominios podrían estar involucrados en la replicación del viroide mediada por la interacción con la polimerasa que cataliza la replicación (Goodman *et al.*, 1984). En la hebra superior del dominio T_L se encuentra la TCR, que podría estar implicada en este proceso (Koltunow y Rezaian, 1988; Flores *et al*, 1997). Otras hipótesis sobre la función de estos dominios es la que los relaciona con el movimiento del viroide en el huésped (Owens y Hammond, 1990; Hammond, 1994) o con el origen de nuevos viroides a través del intercambio o recombinación de secuencias entre distintos viroides que se encuentran co-infectando una misma planta huésped (Keese y Symons, 1985).

3. Localización Subcelular

Los pimeros ensayos para localizar los viroides dentro de la célula, se realizaron obteniendo fracciones subcelulares mediante centrifugación diferencial, y analizando su contenido en RNA viroidal, así como mediante técnicas de hibridación *in situ* en secciones ultra finas de hojas de plantas infectadas. Con estas aproximaciones se localizó PSTVd, CEVd y HSVd en las fracciones nucleares (Diener, 1971; Semancik *et al.*, 1976; Takashi *et al.*, 1982). Posteriormente combinando técnicas de hibridación *in situ* con la microscopía láser confocal de barrido, se pudo localizar PSTVd específicamente en el nucleolo (Harders *et al.*, 1989; Qi y Ding, 2003). Se han obtenido resultados similares para otros viroides de la familia *Pospiviroidae*, lo que ha permitido generalizar que el núcleo es el sitio de su replicación y acumulación.

En contraste con lo anterior, se ha establecido que en hojas de plantas infectadas con ABSVd o con PLMVd, el 80% del RNA viroidal se localizaba en el cloroplasto y más

concretamente en las membranas tilacoidales (Mohamed y Thomas, 1980; Lima *et al*., 1994; Bonfigliogi *et al*., 1994, Bussière *et al*., 1999).

4. Replicación

El mecanismo de replicación de los viroides sigue el modelo de círculo rodante debido a las características de circularidad de sus RNAs (Branch y Dickson, 1980). De acuerdo con dicho modelo, los intermediarios de la replicación son RNAs diferentes del RNA genómico y se acumulan a una concentración inferior a la de éste (Branch y Robertson, 1984; Ishikawa *et al*., 1984; Hutchins *et al*., 1985). El RNA circular monomérico infectivo más abundante se le asigna de manera arbitraria la polaridad positiva (+), y es reconocido por una RNA polimerasa que después de varios ciclos de elongación da lugar a un oligómero de polaridad complementaria (-). Este oligómero a su vez sirve de molde para la síntesis de nuevos oligómeros de polaridad positiva (+) que después son cortados y ligados mediante ribonucleasas y ligasas dando lugar a monómeros circulares de RNA de polaridad (+). Puede seguir dos vías o variantes, simétrica o asimétrica (Figura 2).

En la variante simétrica, el oligómero de polaridad negativa es cortado y ligado obteniéndose monómeros circulares de la misma polaridad y estos a su vez actúan como molde en un segundo ciclo del círculo rodante que da lugar a oligómeros de polaridad positiva (+). La presencia de monómeros circulares de polaridad negativa, indica que la replicación ha seguido la variante simétrica del modelo. Por otro lado en la variante asimétrica, el oligómero de polaridad negativa (-) sirve como molde para la síntesis de oligómeros de polaridad positiva que son luego cortados en monómeros lineales y ligados para dar origen a moléculas circulares maduras de polaridad positiva.

En el caso de los viroides de la familia *Pospiviroidae* y específicamente con PSTVd nunca se han detectado en los tejidos de las plantas infectadas monómeros circulares de polaridad negativa lo que ha llevado a generalizar que todos los miembros de esta familia se replican siguiendo la variante asimétrica (Branch *et al*., 1988). Por otra parte en los estudios realizados con viroides de la familia *Avsunviroidae* si se han identificado RNAs circulares de ambas polaridades, lo cual se ajusta al modelo de la variante simétrica (Hutchins *et al*., 1985; Hernández y Flores, 1992, Navarro *et al.*, 1999). Dado que los intermediarios replicativos del PSTVd se han localizado en el núcleo (Spiesmacher *et al*., 1983) y los del ASBVd en el cloroplasto (Navarro *et al*., 1999), se ha concluido que tanto la replicación como la acumulación del viroide tienen lugar en el núcleo para los viroides de la familia *Pospiviroidae* y en el cloroplasto para los miembros de la familia *Avsunviroidae*.

En la replicación de los viroides, están involucradas tres tipos de enzimas; una RNA polimerasa que sintetiza las moléculas oligoméricas de ambas polaridades, una RNAsa que genera monómeros y una RNA ligasa que los circulariza. Mediante ensayos *in vitro* e *in vivo* utlizando diferentes concentraciones de la α-amanitina (péptido inhibidor de las RNA polimerasas) se demostró que la replicación de PSTVd, CEVd y HSVd se inhibía a concentraciones nanomolares de α-amanitina, lo que llevó a concluir que la RNA polimerasa II era la enzima implicada en la replicación de los viroides de la familia *Pospiviroidae* (Mülbach y Sänger, 1979; Schindler y Mülbach, 1992; Flores y Semancik, 1982; Flores, 1989; Rivera-Bustamante y Semancik, 1989). En los miembros de la familia *Avsunviroidae* participa una RNA polimerasa que no presenta sensibilidad a altos niveles de la α-amanitina (Marcos y Flores, 1992) y por tanto se considera que es diferente a la RNA polimerasa II. Se atribuye pues la replicación a una RNA polimerasa tipo NEP (nuclear encoded polymerase) codificada en el núcleo (Navarro *et al.*, 2000; Navarro y Flores, 2000).
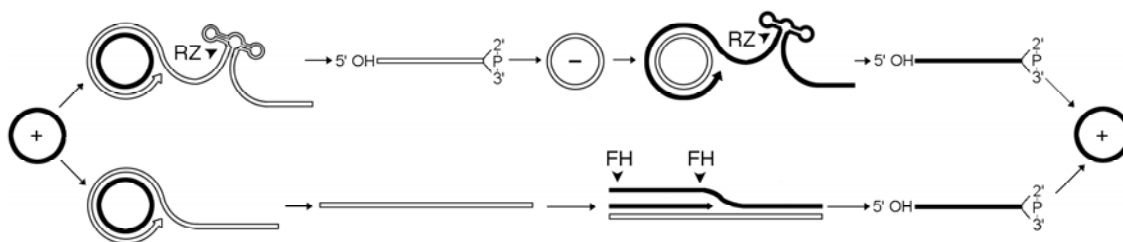
Fig. 2. Modelo de círculo rodante con intermediarios de RNA propuesto como modelo de replicación de los viroides (adaptado de Branch y Roberston, 1984; Symons, 1992; Flores *et al*., 1997). En la parte superior de la figura se representa la variante simétrica del modelo en la que los oligómeros viroidales se autocortan ribozimáticamente (RZ). En la parte inferior se muestra la variante asimétrica donde sólo opera un círculo rodante y un factor del huésped (FH) procesaría las moléculas oligoméricas**.**

En los viroides de la familia *Avsunviroidae* los RNAs de ambas polaridades son capaces de autocortarse *in vitro* a través de sus propias estructuras de cabeza de martillo (Hutchins *et al*., 1986; Hernández y Flores, 1992; Navarro y Flores, 1997) y existen evidencias que indican que probablemente ello suceda también *in vivo* (Symons, 1989; 1992; 1997; Darós *et al*., 1994; Navarro y Flores, 1997). En el proceso de ligación se desconoce si participa una RNA ligasa de localización cloroplástica o son los propios monómeros los que tienen capacidad de autoligarse (Revisado por Flores *et al*., 2005b).

En los viroides de la familia *Pospiviroidae,* para el procesamiento de los oligómeros de polaridad positiva debe participar enzima con actividad RNAsa responsable de reconocer una estructura secundaria concreta del oligómero y realizar cortes específicos (Tsagris *et al*.,

1987; Tsagris *et al*., 1991; Baumstark y Riesner, 1995; Baumstark *et al*., 1997). En la formación de las moléculas circulares completas debe participar una RNA ligasa pero se desconoce si las actividades RNasa y RNA ligasa residen en una misma enzima o en enzimas distintas (Tsagris *et al.*, 1987; Tabler e*t al*., 1992). Mediante experimentos *in vitro* se ha logrado circularizar moléculas lineales monoméricas de PSTVd con una RNA ligasa de germen de trigo (Branch *et al*., 1982).

5. Movimiento y distribución en la planta

Los viroides pueden interactuar directamente con factores del huésped para su movilidad. La infección sistémica de un viroide en una planta huésped ocurre en varias etapas. Primero se produce la entrada dentro de una célula huésped en la cual debe alcanzar el sitio de replicación (el núcleo en los miembros de la familia *Pospiviroidae* o el cloroplasto en los miembros de la familia *Avsunviroidae*), a continuación la invasión de células adyacentes por parte de la progenie resultante, y finalmente el viroide debe moverse de un órgano a otro hasta invadir la planta completamente (Ding y Owens, 2003). A pesar de la información disponible acerca del movimiento del viroide en la planta, los mecanismos básicos implicados aún no han sido dilucidados.

Movimiento intracelular: Después de entrar en una célula, el RNA viroidal debe moverse hasta su sitio de replicación bien sea el núcleo o el cloroplasto. Los ensayos realizados con protoplastos permeabilizados, han mostrado que la importación nuclear del PSTVd es un proceso dependiente de un motivo de estructura primaria o secundaria ya que fragmentos de mRNAs no seguían la misma pauta (Woo *et al*., 1999). Estos resultados y los obtenidos empleando un vector viral que expresaba la proteína verde fluorescente (GFP) y que contenía un intrón con la secuencia completa del PSTVd (Zhao *et al*., 2001) sugieren que para que el viroide entre en el núcleo se debe formar un complejo ribonucleoproteico (Revisado por Flores *et al*., 2005b).

El mecanismo por el cual los miembros de la familia *Avsunviroidae* llegan al cloroplasto no ha sido todavía investigado y de hecho no se ha descrito ningún tipo de movimiento de RNAs que invadan o salgan del cloroplasto (Ding *et al.,* 1999), ni si los viroides son capaces o no de replicarse en otros tipos de plastidios (Ding e Itaya, 2007).

Movimiento intercelular: Una vez el viroide ha infectado la primera célula puede colonizar las células adyacentes y a partir de éstas invadir las partes más distales de la planta. Posiblemente las proteínas y los ácidos nucleicos endógenos o de origen viral se mueven de célula a célula por medio de los plasmodesmos, ya que éstos ofrecen conexiones citoplasmáticas entre células adyacentes (Revisado por Flores *et al*., 2005b). Los estudios con

PSTVd indican que los viroides de esta familia siguen esta ruta para el movimiento intercelular (Ding *et al.,* 1997).

Movimiento a larga distancia: El movimiento sistémico del viroide ocurre a través del sistema vascular, donde se desplaza hacia tejidos y órganos que se encuentran alejados del sitio inicial de la infección, al igual que sucede con los virus. Este transporte conlleva la descarga del viroide al floema y siguiendo el flujo de los fotoasimilados se introduce en otros órganos de la planta. El movimiento de PSTVd se ha estudiado por medio de hibridación *in situ*, con lo que se demostró la presencia del viroide en tejidos vasculares del tallo y las raíces de plantas de tomate (Hammond, 1994; Stark-Lorenzen *et al.*, 1997). El viroide del cadang-cadang del cocotero (*Coconut cadang-cadang viroid*, CCCVd) y CEVd también se han encontrado en tejidos vasculares de plantas infectadas (Bonglifioli *et al.*, 1996).

6. Clasificación Taxonómica

La clasificación taxonómica de los viroides se fundamenta en los análisis filogenéticos que han sido elaborados a partir de las secuencias completas y de los motivos conservados de los mismos (Elena *et al.*, 1991; 2001). Las 29 especies de viroides se agrupan en dos familias (Tabla 2) de acuerdo con tres criterios.

Los viroides de la familia *Pospiviroidae*, cuya especie tipo es PSTVd, tienen las siguientes características: (i) tienen CCR; (ii) carecen de autocorte mediado por estructuras ribozimáticas; y (iii) se replican y acumulan en el núcleo mediante la vía asimétrica del círculo rodante. Los viroides de la familia *Avsunviroidae*, cuya especie tipo es ABSVd, tienen las siguientes características: (i) carecen de CCR; (ii) poseen actividad ribozimatica mediada por estructuras de cabeza de martillo; (iii) se replican y acumulan en el cloroplasto siguiendo el modelo simétrico del círculo rodante.

Dentro de cada familia, los viroides están agrupados en géneros (Tabla 2), de acuerdo a diferentes criterios. En la familia *Pospiviroidae*, la secuencia de la CCR y la presencia o ausencia de dos motivos conservados (TCR o TCH) (Koltnow y Rezaian, 1988) son los criterios para ubicar un viroide dentro de su género. Dentro de esta familia se diferencian cinco géneros con TCR, *Pospiviroid, Apscaviroid* y *Coleoviroid. Hostuviroid* y *Cocadviroid* con TCH. En la familia *Avsunviroidae* cuyos miembros carecen de CCR y de otros motivos conservados (TCR o TCH), los géneros *Avsunviroid, Pelamoviroid* y *Elaviroid* se han definido en base al contenido en G+C, a la estructura de mínima energía libre, al tipo de estructura de cabeza de martillo y a la solubilidad en LiCl 2M.

La diferenciación a nivel de especie se ha establecido de manera arbitraria de forma que dos viroides con una identidad de secuencia inferior al 90% se consideran como especies distintas, mientras que cuando ésta es superior al 90% se consideran como variantes de la misma especie. Sin embargo, en la actualidad la ICTV recomienda tener también en cuenta las propiedades biológicas, específicamente hospedadores naturales y experimentales como un segundo criterio para su clasificación en géneros y especies. Las actualizaciones y las descripciones relacionadas con la taxonomía de los viroides se encuentran en: www.ictvonline.org/virusTaxonomy.asp.

Tabla 2. Clasificación de los viroides (adaptado con modificaciones de Elena *et al*., 1991; Hernández y Flores, 1992; Flores *et al*., 1998; 2000).

FAMILIA	GENERO	ESPECIE
Pospiviroidae	*Pospiviroid*	**PSTVd (*Potato spindle tuber viroid*)**
		TCDVd (*Tomato chlorotic dwarf viroid*)
		MPVd (*Mexican papita viroid*)
		TPMVd (*Tomato planta macho viroid*)
		CSVd (*Chrysanthemum stunt viroid*)
		CEVd (*Citrus exocortis viroid*)
		TASVd (*Tomato apical stunt viroid*)
		IrVd (*Iresine viroid 1*)
		CLVd (*Columnea latent viroid*)
	Hostuviroid	**HSVd (*Hop stunt viroid*)**
	Cocadviroid	**CCCVd (Coconut cadang-cadang viroid)**
		CTiVd (*Coconut tinangaja viroid*)
		HLVd (*Hop latent viroid*)
		CBCVd (*Citrus bark cracking viroid*)
	Apscaviroid	**ASSVd (Apple scar skin viroid)**
		CDVd (Citrus dwarfing viroid)
		ADFVd (*Apple dimple fruit viroid*)
		GYSVd-1 (*Grapevine Yellow speckle viroid 1*)
		GYSVd-2 (*Grapevine Yellow speckle viroid 2*)
		CBLVd (*Citrus bent leaf viroid*)
		PBCVd (*Pear blister canker viroid*)
		AGVd (*Australian grapevine viroid*)
	Coleviroid	**CbVd-1 (*Coleus blumei viroid 1*)**
		CbVd-2 (*Coleus blumei viroid 2*)
		CbVd-3 (*Coleus blumei viroid 3*)
Avsunviroidae	*Avsunviroid*	**ASBVd (*Avocado sunblotch viroid*)**
	Pelamoviroid	**PLMVd (*Peach latent mosaic viroid*)**
		CChMVd (*Chrysanthemun chlorotic mottle viroid*)
	Elaviroid	***ELVd (Eggplant latent viroid)***

La familia *Pospiviroidae* contiene cinco géneros: *Pospiviroid*, *Hostuviroid*, *Cocadviroid*, *Apscavairoid* y *Coleoviroid*.

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Al género *Pospiviroid* pertenecen nueve viroides: PSTVd, CEVd, CSVd, *Tomato chlorotic dwarf viroid* (TCDV), *Tomato planta macho viroid*, (TPMVd), *Mexican papita viroid,* (MPVd), *Tomato apical stunt viroid,* (TASVd), I*resine viroid I* (IrVd) y *Columnea latent viroid* (CLVd). PSTVd es la especie tipo de este género. La gama de huéspedes naturales incluye papa, tomate (Puchta *et al*., 1990; Elliot *et al*., 2001; Verhoeven *et al*., 2004), aguacate (Querci *et al*., 1995), pepino (Shamloul *et al*., 1997) y solanáceas ornamentales como *Solanum jasminoides*, V*erbena sp*, *Impatiens sp* (Verhoeven *et al.,* 2008; Bostan *et al*., 2004; Nie *et al*., 2005; Singh *et al*., 2006), y se ha logrado transmitir a una amplia gama de huespedes herbáceos experimentales que incluye al menos 156 especies de 12 familias diferentes (Diener, 1979). CEVd que es miembro de este mismo género, y además de infectar distintas especies de cítricos, en la mayoría de casos sin producir síntomas, coloniza de forma natural muchas otras especies en las que puede causar enfermedades o encontrarse como infecciones latentes. CEVd puede infectar y replicarse en solanáceas y en especies de la familia *Compositae* (Durán-Vila *et al*., 1986; García-Arenal *et al*., 1987; Mishra *et al*., 1991; Fagoaga y Durán-Vila, 1996, Fadda *et al*., 2003b). Con la excepción del CSVd que se tratará mas adelante, TCDV, MPVd, TPMVd y TASVd han causado solamente problemas esporádicos. En general los miembros de este género pueden producir síntomas similares en determinados huéspedes como el tomate, que ha sido y continúa siendo muy utilizado como un huésped experimental por su fácil manejo y los periodos de incubación relativamente cortos para manifestar síntomas. Los síntomas característicos son enanismo, epinastia, distorsión y/o decoloración de hojas y necrosis, usualmente de venas pero algunas veces de peciolo y tallo. Estos viroides se pueden transmitir con facilidad por medios mecánicos, abrasión e inoculación por injerto (Singh y Ready, 2003).

El género *Hostuviroid* es un género monotípico cuyo único miembro es HSVd. Este viroide tiene una amplia gama de huéspedes naturales y experimentales. Como infección natural se ha descrito en lúpulo (Sasaki y Shikata, 1977), cítricos (Sano *et al*., 1988), pepino (Van Dorts y Peters, 1974), almendro (Cañizares *et al*., 1999), ciruelo (Sano *et al*., 1989), melocotonero (Sano *et al.*, 1989), peral (Shikata, 1990) y albaricoquero y almendro (Astruc *et al*., 1996; Cañizares *et al*., 1999). La infección puede presentarse de forma latente en algunos hospedadores como la vid (Shikata, 1990; Polivka *et al*., 1996) y el albaricoquero (Astruc *et al*., 1996). Las variantes de secuencia de HSVd se han dividido filogenéticamente en tres grupos que corresponden a los tipos "Citrus", "Plum" (ciruelo) y "Hop" (lúpulo) (Shikata, 1990; Astruc *et al*., 1996). En el tipo "Plum" se ubican las variantes procedentes de aislados de melocotonero, ciruelo y vid; en el tipo "Hop" las de aislados de lúpulo, vid, melocotonero y peral, y en el tipo "Citrus" las de aislados de pepino y cítricos (Sano *et al*., 1989; Shikata, 1990).

En el género *Cocadvirod* se encuentran cuatro viroides: CCCVd, el viroide del tinangaja del cocotero (*Coconut tinangaja viroid*, CTiVd), el viroide latente del lúpulo (*Hop latent viroid*, HLVd) y el viroide de la corteza agrietada de los cítricos (*Citrus bark cracking viroid*, CBCVd). CCCVd y CTiVd infectan solo a especies de la familia *Palmae*. CCCVd se ha detectado también en la palma de aceite (*Elaeais guineensis)* y otras monocotiledóneas que crecen de forma silvestre en Filipinas (Flores y Duran-Vila, 1986). Además se ha logrado transmitir experimentalmente tanto a esta especie como a la palma datilera (*Phoenix dactylifera)* entre otras (Randles y Rodríguez, 2003). El HLVd es un viroide muy difundido en lúpulo en la mayoría de zonas productoras del mundo (Barbara *et al*., 1990) incluida España (Pallás *et al*., 1987). Aunque su nombre indica que no induce síntomas, tiene un efecto importante en plantaciones de lúpulo de Gran Bretaña (Adams *et al*., 1992) causando en algunas variedades una reducción del vigor y del contenido de alfa ácidos (Barbara *et al*., 1990). CBCVd también ubicado en este género, es uno de los viroides menos diseminados de los que afectan a los cítricos. Este ha sido identificado en California y ha sido asociado a factores enanizantes transmisibles por injerto en Israel y Turquía (Hadas *et al*., 1989; Önelge *et al*., 2000). CBCVd ha sido incluido recientemente por el ICTV, como una nueva especie del género *Cocadviroid* por presentar un subconjunto de nucleótidos de la CCR similar al de CCCVd así como la TCH del dominio T_L típica de los miembros de este género (Flores *et al.*, 1998; 2000). Sin embargo, no se tuvo en cuenta que este viroide posee una región de 80 a 90 nucleótidos localizados en los dominios V y T_R idéntica a la de CEVd (Puchta *et al.*, 1991), por lo que dada la relación entre estos dos viroides, algunos autores han sugerido que CBCVd debería haberse ubicado en el genero *Pospiviroid* (Semancik y Vidalakis, 2005).

En el género *Apscaviroid*, se incluyen ocho viroides que infectan especies frutales: el viroide de la piel cicatrizada de la manzana (*Apple scar skin viroid*, ASSVd), el viroide del enanismo de los cítricos (*Citrus dwarfing viroid*, CDVd), ADFVd, el viroide 1 del moteado amarillo de la vid (*Grapevine yellow speckle viroid 1*, GYSVd-1), el viroide 2 del moteado amarillo de la vid (*Grapevine yellow speckle viroid 2*, GYSVd-2), el viroide de la hoja curvada de los cítricos (*Citrus bent leaf viroid*, CBLVd), el PBCVd y el viroide australiano de la vid (*Australian grapevine viroid*, AGVd). ASSVd es la especie tipo de este género. Dentro de este género se encuentran CBLVd y CDVd (anteriormente conocido como *Citrus viroid III,* CVd-III), que afectan a los cítricos. Recientemente se han descrito otros dos viroides denominados viroide de los cítricos OS (*Citrus viroid OS*, CVd-OS) (Ito *et al*., 2001) y viroide V de los cítricos (*Citrus viroid V*, CVd-V) (Serra *et al*., 2008b), que aunque no han sido todavía aceptados por el Comité Internacional de Taxonomía de Virus (ICTV), presentan las propiedades biológicas y moleculares características de los miembros de este género. Todos estos viroides que infectan especies perennes han sido poco estudiados, debido principalmente

a que no se dispone de hospedadores herbáceos experimentales de fácil manejo necesarios para su estudio biológico.

La familia *Avsunviroidae* contiene tres géneros: *Avsunviroid*, *Pelamoviroid* y *Elaviroid*.

Al género *Avsunviroid,* pertenece exclusivamente ASBVd. La transmisión natural y la expresión de síntomas esta limitado al aguacate (*Persea americanum*). En condiciones experimentales se ha logrado transmitir por injerto a otros miembros de la familia *Lauracea* como *Persea shiedeana*, *Cinnamomum zeylanicum*, *Cinnamomum camphora* y *Ocotea bullata*. Los intentos realizados para transmitirlo a especies herbáceas indicadoras de otros viroides como tomate y *Gynura aurantiaca* no han dado resultados satisfactorios (Semancik, 2003).

Dentro del género *Pelamoviroid* se han agrupado PLMVd y CChMVd. Estos dos viroides están estrechamente relacionados y comparten características similares en cuanto a tamaño, estructuras secundarias muy ramificadas e insolubilidad en LiCL 2M. PLMVd causa la enfermedad del mosaico latente del melocotonero que es exclusiva de esta especie y de sus híbridos. Aunque se han hecho intentos para trasmitir el viroide a otros huéspedes, incluidos otros frutales del género *Prunus*, los resultados han sido negativos (Desvignes, 1986).

CChMVd tiene una gama reducida de huéspedes en la que se incluyen algunos cultivares de crisantemo que son susceptibles. De las 51 especies y cultivares evaluados como posibles huéspedes, incluyendo 9 especies de *Chrysanthemum moriflorum*, solo la especie *C. moriflorum* ahora denominada *Dendranthema grandiflora* cv. 'Bonnie Jean', 'Deep Ridge', 'Yellow Delaware', *Ch. zawadskii* var. l*atibolium* cv. 'Clara Curtiss' fueron susceptibles (Flores *et al.*, 2003).

Al género *Elaviroid* pertenece ELVd. Este viroide fue identificado inicialmente en plantas asíntomáticas de berenjena, cultivar 'Sonja'. Se demostró que otros cultivares de berenjena también eran susceptibles a la infección pero no manifestaban síntomas. Las pruebas de infectividad realizadas en tomate, pepino, crisantemo y cidro dieron resultados negativos (Fagoaga, 1995). La reducida gama de huéspedes, baja estabilidad y recuperación en LiCL 2M y los resultados negativos de las hibridaciones realizadas con ribosondas generadas a partir de secuencias de ASBVd, ASSVd, CEVd y HSVd (Fagoaga y Duran-Vila, 2003), sugirieron que se trataba de un nuevo miembro de la familia *Avsunviroidae* (Fadda *et al*., 2003a).

7. Variabilidad de los viroides

Los viroides por tener genomas de RNA, se encuentran sujetos a una elevada tasa de variabilidad genética; debido a que se replican por medio de una RNA polimerasa dependiente

de DNA que tiene una alta tasa de error ya que carece de mecanismos de prueba de lectura. Basándose en esta característica se ha propuesto un modelo que explica como la replicación de los genomas de RNA generan poblaciones de secuencias mutantes denominadas cuasi-especies, en la que las secuencias mutantes difieren de una o varias secuencias predominantes (Domingo *et al*., 1996; Holland *et al*, 1982).

Dentro de un aislado se han encontrado diferentes variantes de secuencia en PSTVd (Lakshman y Tavantzis, 1992), CEVd (Visvader y Symons, 1985; Gandía *et al*., 2007), HSVd (Palacio-Bielsa *et al.,* 2004), CBLVd (Gandía y Duran-Vila, 2004) o PLMVd (Hernández y Flores, 1992; Di Serio, 1995; Ambrós *et al*., 1998), entre otros. La existencia de diferentes variantes de secuencia dentro de una planta puede ser consecuencia de distintos eventos de inoculacion/infección y además el resultado de la variabilidad generada durante el propio proceso de replicación de los viroides. La disponibilidad de metodologías para inocular secuencias individuales mediante la síntesis de transcritos diméricos ha permitido demostrar que una única secuencia es capaz de generar poblaciones mas o menos heterogéneas de variantes (Gandía y Duran-Vila, 2004; Gandía *et al.,* 2005) y que en estas poblaciones coexisten variantes (o haplotipos) capaces de infectar huéspedes sensibles de forma individual y de manifestar distintos grados de virulencia (Visvader y Symons, 1985; Lakshman y Tavantzis, 1993).

Los huespedes de viroides e incluso los tejidos que los albergan juegan un papel importante en la selección de variantes que se encuentran en una planta infectada (Semancik *et al*., 1993; Semancik *et al*., 1994). Utilizando el CEVd como modelo, se ha demostrado que el huésped actúa como presión de selección sobre la estructura de la población (variantes y frecuencias del viroide) (Bernad *et al*., 2009). Además, la caracterización de un aislado suave y otro agresivo de CEVd inoculados en *G.aurantiaca* y tomate, mostró que las variantes recuperadas del aislado agresivo conservaban las características de las razas agresivas, pero una de las variantes recuperadas del aislado suave inducía síntomas agresivos (Chaffai *et al.,* 2007).

Dado el efecto del huésped en la composición y estructura de la población de viroides, las secuencias disponibles en las bases de datos hay que tomarlas con cautela, ya que la utilización de hospederos herbáceos para la secuenciación de viroides puede no reflejar la composición del aislado en el huésped natural del cual provenía. Con las técnicas actuales de RT-PCR se pueden caracterizar los aislados en los huéspedes originales/naturales y secuenciandolos diferenciar de manera más precisa las distintas variantes de secuencia que lo componen.

8. Enfermedades Producidas por Viroides

Los viroides son entidades biológicas que despiertan interés en el ámbito de la investigación básica y aplicada. En fitopatología el interés radica en el hecho de que actúan como agentes causales de enfermedades de importancia económica. Los viroides infectan y se replican únicamente en plantas superiores tanto monocotiledóneas como dicotiledóneas afectando a especies leñosas y/o herbáceas. Su nombre científico se refiere a la especie en la cual se han identificado por primera vez y a los síntomas asociados. La gama de huéspedes de los viroides es variable, pudiendo ser muy restringida como en el caso de ASBVd, CCCVd, PLMVd y ELVd (Desjardins, 1987; Desvignes, 1986; Dimock *et al*., 1971; Fadda *et al*., 2003) o muy amplia como el caso de CEVd, (Durán-Vila *et al*., 1986; García-Arenal *et al*., 1987; Mishra *et al*., 1991; Fagoaga y Durán-Vila, 1996) y HSVd (Shikata, 1990; Astruc *et al*., 1996). La mayoría de viroides que presentan una gama de huéspedes amplia y en particular CEVd y HSVd, pueden presentarse como infecciones latentes en especies tolerantes o causando enfermedades en especies sensibles.

Los síntomas que inducen las infecciones viroidales no son específicos y no se diferencian claramente de los producidos por virus. De hecho antes de descubrir los viroides, a las enfermedades causadas por este tipo de patógenos se les atribuía una etiología viral, basandose en la transmisión por injerto. Se han descrito distintos síntomas atribuidos a la infección viroidal, uno común es el enanismo asociado al acortamiento de los entrenudos, síntomas foliares de rugosidad, epinastia, moteados cloróticos y necrosis de hojas. En plantas de papa infectadas con PSTVd, además de observarse síntomas en hojas y tallos, los tubérculos también se encuentran afectados y son de menor tamaño, forma alargada o fusiforme, ojos abundantes y prominentes, y en algunos casos grietas (Singh *et al*., 2003). En pepino cultivado en invernadero los frutos de las plantas infectadas con HSVd tienen un tamaño reducido y son de color verde pálido (Singh *et al*., 2003), de aquí el nombre "enfermedad del fruto pálido del pepino" que se atribuyó a un nuevo viroide al que se denominó viroide del fruto pálido del pepino (*Cucumber pale fruit viroid*, CPFVd) hasta que al secuenciarse se demostró que se trataba de una variante de HSVd.

En especies leñosas el efecto de la infección viroidal en general se desarrolla de forma lenta y las plantas pueden incluso tardar varios años en manifestar síntomas, como en el caso de palmas de coco infectadas con el viroide CCCVd, son de menor tamaño y la producción de frutos se ve mermada (Randles y Rodríguez, 2003). En cítricos los síntomas descritos como consecuencia de infecciones viroidales se caracterizan por la falta de vigor de los árboles y por la presencia de lesiones, descamaciones y acanaladuras de la madera, y proyecciones en la cara

cambial de la corteza acompañadas por exudaciones o impregnaciones de goma. Más adelante se dará una descripción más detallada de los efectos de los viroides en cítricos.

Otro viroide que induce síntomas característicos en leñosas es ASBVd que infecta aguacate, provocando hendiduras en el tallo, zona deprimidas de color blanco, amarillo o púrpura en ramas viejas y decoloraciones en el fruto (Semancik *et al*., 2003). PLMVd tambien infecta especies leñosas. Afecta sobre todo la calidad del fruto del melocotonero, ocasionalmente produce alteraciones en el follaje, como mosaicos cloróticos que en casos muy agresivos pueden cubrir la mayor parte de la hoja. Los síntomas aparecen dos años después de plantar el material infectado, que puede manifestar retraso en la floración, brotación y maduración de los frutos. El efecto en los frutos se manifiesta como malformaciones, decoloraciones, grietas en la línea de sutura y semillas aplanadas (Flores *et al.,* 2003).

Las vides afectadas por GYSVd-1 y/o GYSVd-2 presentan síntomas foliares de amarilleo que son difíciles de caracterizar ya que pueden manifestar manchas muy pequeñas en una o dos hojas e incluso hasta un moteado que puede afectar toda la hoja (Little y Rezaian, 2003). En realidad estos viroides no producen daños económicos, pero las vides infectadas son más vulnerables a los daños causados por el virus del entrenudo corto infeccioso (*Grapevine fanleaf virus*, GFLV), y pueden llegar a manifestar el síndrome conocido como "Big vein banding" como consecuencia de un efecto sinérgico entre ambos patógenos(Semancik y Szykowski, 1992; Szykowski *et al.*, 1995).

La expresión de síntomas, esta determinada por el huésped y las condiciones ambientales en las que se desarrollan las plantas infectadas, ya que temperaturas relativamente altas (30-33$^{\mathrm{o}}$C) y elevadas intensidades de luz favorecen la replicación y acumulación del viroide (Sänger y Ramm, 1974; Singh, 1983; 1989). Otros factores que modulan la expresión de síntomas son la virulencia del aislado, la co-infección con otros agentes patógenos y el estado nutricional de la planta.

A continuación y antes de describir las enfermedades producidas por viroides en cítricos que constituyen el objetivo de esta tesis, se describen de manera somera cuatro enfermedades de origen viroidal que podrían afectar a cultivos de interés comercial en Colombia.

8.1. La enfermedad del tubérculo fusiforme de la patata

Está causada por PSTVd que es la especie tipo del genero *Pospiviroid* dentro de la familia *Pospiviroidae.* PSTVd, con un tamaño que oscila entre 356-360 nucleótidos (Gross *et al.,* 1978), ha sido ampliamente estudiado y presenta una gama de huespedes experimentales

mayor que otros viroides. Infecta diversas especies solanáceas, como la papa y el tomate; otras especies susceptibles que se mencionan en la literatura científica son el pepino y algunas especies de la familia de las compuestas. Los síntomas causados por PSTVd en papa incluyen cambios en el color de la hoja, retraso en la brotación (Figura 3A) y en infecciones intensas causan necrosis en peciolos, tallos principales y enanismo de la planta (Singh *et al*., 2003). Los tubérculos de las plantas afectadas muestran chancros y una elongación excesiva con lo que se ve mermado su valor comercial (Figura 3B). Su efecto sobre la producción se manifiesta en la reducción de número y peso de los tubérculos que puede alcanzar entre un 17-24% en el caso plantas infectadas con aislados suaves y hasta un 64% en el caso de plantas infectadas con aislados agresivos (Sinhg *et al.,* 2003). La difusión de la enfermedad tiene lugar fundamentalmente mediante la propagación de material infectado, aunque PSTVd se transmite también por vía mecánica (maquinaria y herramientas contaminadas), semilla y polen. Actualmente se ha observado que PSTVd se halla muy difundido en solanáceas ornamentales como *Solanum jasminoides*, *S. rantonnetii* y varias especies de *Brugmansia* (*B. suaveolens, B. candida, B. cordata* y *B. variegata*) (Verhoeven *et al*., 2004; Di Serio, 2007; Verhoeven *et al.,* 2008), lo que ha puesto en alerta a las autoridades fitosanitarias europeas ya que PSTVd es un organismo de cuarentena en la Unión Europea.

Fig. 3. Síntomas inducidos por PSTVd en plantas de patata. (derecha) A) aspecto general de la planta. B) efecto sobre los tubérculos. Planta y tubérculo sanos (izquierda) e infectados (derecha). Foto cedida por P.R. Desjardins (Universidad de California, Riverside).

En PSTVd, la secuencia del dominio patogénico determina la expresión de síntomas y solo se requiere la substitución de 3 a 4 nucleótidos en la hebra inferior del dominio P para convertir una raza intermedia de PSTVd en una raza agresiva (Schnölzer *et al*., 1985; Owens *et al*., 1996).

8.2. La enfermedad del manchado solar del aguacate

Está producida por ASBVd que es la especie tipo del genero *Avsunvirod* dentro de la familia *Avsunviroidae*. ASBVd posee entre 246-250 nucleótidos (Symons, 1981) y su

estructura secundaria no se ajusta al modelo de los dominios estructurales propuestos por Keese y Symons (1985) pero puede formar estructuras ribozimáticas de cabeza de martillo con propiedad de autocorte (Hutching *et al*., 1986). La gama de huespedes esta limitada a la familia de las lauráceas y en particular a la especie *Persea americana* (Semancik y Szychowski, 1994). Se transmite a través del polen, semilla y por injerto de raíces. No se han identificado insectos vectores.

La enfermedad se ha caracterizado por el desarrollo de diferentes síntomas que incluyen estrías en el tallo, decoloraciones de color blanco en tallos y brotes; y depresiones de color amarillo o púrpura de tamaños diferentes en frutos (Figura 4). En hojas se han descrito dos patrones de síntomas, uno caracterizado por amplias zonas cloróticas asociadas al tejido vascular o a zonas próximas y el otro más generalizado (Desjardins, 1987).

Fig. 4. Síntomas de ASBVd en frutos de aguacate. Foto cedida por P.R. Desjardins (Universidad de California, Riverside).

En condiciones experimentales, se monitorearon árboles infectados con ABSVd y se definieron dos patrones de síntomas foliares, uno se presentó como intensas zonas cloróticas asociadas con el pecíolo y el tejido vascular y el otro como una variegación que se extendía a lo largo de la hoja. Los síntomas foliares se dividieron en tres tipos; decoloración, variegación y asintomáticos. En este complejo de síntomas se identificaron variantes de secuencia denominadas ASBVd-B (asociada a tejidos que presentaban decoloración), ASBVd-V (asociada a la variegación) y ASBVd-Sc (asociada a tejidos asintomáticos). Estas variantes presentaban tamaños de 247 a 250 nucleótidos. Las variantes de mayor tamaño (249-250 nt) correspondían a las variantes ASBVd-B mientras que en las ASBVd-V y ASBVd-Sc se encontraron variantes de menor tamaño (247-248 nt) (Semancik y Szychowski, 1994).

8.3. La enfermedad del Cadang-Cadang del cocotero

Está asociada con CCCVd que es la especie tipo del genero *Cocadviroid* dentro de la familia *Pospiviroidae* (Flores *et al*., 2000). Aunque no se han completado los postulados de Koch, la asociación entre la enfermedad y CCCVd indica que éste es el agente causal de la

misma. CCCVd, con 246 nucleótidos, es el agente infeccioso viroidal más pequeño que se conoce. El desarrollo de la enfermedad en palma se ha caracterizado en tres estadios diferentes (temprano, intermedio y tardío) de acuerdo con la evolución de síntomas (Figura 5) que está acompañada con cambios en el tamaño y estructura del viroide. Durante las primeras fases del estadio temprano, cuando las palmas son todavía asintomáticas, se identifica el viroide de 246-247 nucleótidos. Al cabo de 1-2 años los cocos producidos por estas palmas tienen una forma mas redondeada que los normales y presentan lesiones en la zona ecuatorial aunque las hojas siguen sin manifestar síntomas. El desarrollo posterior de síntomas se caracteriza por la aparición de manchas cloróticas en hojas, inflorescencias con necrosis en las puntas, pérdidas de flores masculinas y se detectan formas más grandes de viroide con tamaños de 296 y 301 nucleótidos. En el estadio intermedio de la enfermedad los síntomas se hacen más intensos y hay una disminución drástica de la producción y solo se detectan las formas de mayor tamaño del viroide. Finalmente en el último estadio las hojas se vuelven quebradizas, presentan una clorosis generalizada, la corona es de menor tamaño y la palma muere. La gama de huéspedes esta restringido a la familia *Palmae*. Se puede transmitir el viroide de forma experimental mediante inyección de extracto infectado. Aunque la difusión de la enfermedad en campo sugiere la implicación de algún vector, éste no ha sido identificado.

Fig. 5. Palmeral afectado por la enfermedad del "cadang-cadang". Muestra palmeras en los estadíos temprano, intermedio y final de los síntomas de la enfermedad. Foto tomada de *Invasive.org*

8.4. La enfermedad del enanismo del crisantemo

Está producida por CSVd que pertenece al género *Pospiviroid* dentro de la familia *Pospiviroidae*. Las variantes de este viroide tienen un tamaño entre 354 y 356 nucleótidos (Haseloff y Symons, 1981) y afecta al crisantemo, un cultivo ornamental estacional con un impacto económico bien documentado. El desarrollo de los síntomas depende de las condiciones de crecimiento de las plantas y de la sensibilidad de la variedad. Se han descrito variedades que se comportan como portadoras asintomáticas del viroide. Aunque la expresión

de síntomas es variable, la más común es la reducción del tamaño de las plantas que presentan entrenudos cortos, la lámina foliar menos desarrollada y con lesiones cloróticas (Figura 6). Las flores presentan también tamaño reducido (Bouwen y Van Zaayen, 2003), color más pálido, y se abren aproximadamente de 7 a 10 días antes que las flores de las plantas no afectadas. Todo ello se traduce en que en algunos países como España suponga la pérdida casi total de su valor comercial. CSVd se difunde fundamentalmente mediante importaciones de material propagativo y en cultivos de invernadero mediante transmisión mecánica. No se ha demostrado la transmisión mediante vectores y los informes acerca de la transmisión por semilla son contradictorios.

Fig. 6. Síntomas producidos por CSVd (izquierda) en plantas de crisantemo "Bonnie Jean".

9. Viroides de los Cítricos

Los primeros estudios sobre los viroides de los cítricos se iniciaron cuando se logró demostrar que la enfermedad de la exocortis de los cítricos, antes atribuida a un virus, era causada por un nuevo agente infeccioso al que se denominó Citrus exocortis viroid (Semancik y Weathers, 1972). Incluso antes de establecer la naturaleza viroidal de este patógeno, para el diagnóstico de la enfermedad se utilizaban métodos biológicos empleando el cidro Etrog (*Citrus medica* L.) como planta indicadora. La transmisión por injerto desde árboles generalmente asintomáticos a esta especie indicadora podía provocar una gama de síntomas que oscilaba desde muy suaves a muy agresivos (Calavan, 1968; Roistacher *et al.,* 1977), y durante mucho tiempo se consideró equivocadamente que estas distintas reacciones eran debidas a la existencia de distintas razas de CEVd.

Posteriormente, con el desarrollo de un sistema de doble electroforesis (electroforesis secuencial en geles de poliacrilamida sPAGE), la primera realizada en condiciones no

desnaturalizantes y la segunda en geles que contenían urea y por tanto provocaban la desnaturalización de los RNAs, se descubrió que los cítricos podían ser portadores de otros RNAs con movilidades eletroforéticas distintas a la de CEVd. Las condiciones de electroforesis empleadas permitieron establecer que estos RNAs distintos a CEVd, eran también circulares y estaban presentes en diferentes aislados de campo (Duran-Vila *et al.*, 1986; Duran-Vila *et al.*, 1988). La naturaleza viroidal de estos RNAs circulares se estableció mediante la inoculación de preparaciones obtenidas rescatando cada uno de estos RNAs de los geles en los que se habían separado por sPAGE. Estos viroides se denominaron "Citrus Viroids (CVds)" y fueron clasificados en cinco grupos de acuerdo con su movilidad electroforética en 5% sPAGE, la similitud de secuencia determinada por hibridación con sondas de cDNA específicas de cada viroide, y por los síntomas que provocaban en la indicadora cidro Etrog (Duran-Vila *et al.*, 1988). Estos grupos fueron denominados CEVd, CVd-I, CVd-II, CVd-III y CVd-IV. Con la secuenciación posterior de varias cepas se estableció que en cada grupo se habían ubicado viroides cuyas secuencias estaban relacionadas y presentaban identidades de secuencia superiores al 90%, por lo que de acuerdo con la normativa establecida por ICTV debían ser considerados como variantes de un mismo viroide. En la actualidad algunos han sido renombrados: (i) CBLVd corresponde al viroide que inicialmente se había denominado CVd-I y el nombre se refiere a la curvatura de la hoja de cidro Etrog inducida por este viroide (Ashulin *et al.*, 1991); (ii) HSVd corresponde al nombre de un viroide identificado y caracterizado anteriormente en lúpulo y sustituye al nombre CVd-II ya que las numerosas variantes caracterizadas en cítricos presentan identidades de secuencia superiores al 90% con la secuencia de referencia de HSVd (Levy y Hadidi, 1993; Reanwarakorn y Semancik, 1998); (iii) CDVd corresponde al viroide que inicialmente se había denominado CVd-III y el nombre se refiere al enanismo inducido por este viroide en árboles injertados sobre patrones sensibles al mismo (Vernière *et al.*, 2004); (iv) CBCVd corresponde al viroide que inicialmente se había denominado CVd-IV y el nombre se refiere a las grietas inducidas en la corteza del naranjo trifoliado (*Poncirus trifoliata*) (Vernière *et al.*, 2004), aunque tanto esta nomenclatura como la inclusión de este viroide dentro del género *Cocadviroid* ha sido cuestionada por algunos especialistas (Semancik y Vidalakis, 2005).

En la Tabla 3 se presentan tanto los viroides de cítricos incluidos en el presente sistema de taxonómico de la ICTV, como otros dos viroides, CVd-OS y CVd-V (Ito *et al.*, 2001, Serra *et al.*, 2008b), que conforman un conjunto de siete viroides distintos en cítricos.

Tabla 3. Clasificación de los viroides de los cítricos y las enfermedades que producen

		Viroides de los cítricos		
Familia	**Género**	**Especie**[1]	**Acrónimos**	**Enfermedad**
	Pospiviroid	*Citrus exocortis viroid*	CEVd	Exocortis
	Hostuviroid	*Hop stunt viroid*	HSVd	Caquexia
	Apscaviroid	*Citrus bent leaf viroid*	CBLVd	
Pospiviroidae	*Apscaviroid*	*Citrus dwarfing viroid*	CDVd	
	Apscaviroid	*Citrus viroid V*	CVd-V	
	Apscaviroid	*Citrus viroid OS*	CVd-OS	
	Cocadviroid	*Citrus bark cracking viroid*	CBCVd	

[1] Nomenclatura aceptada por el ICTV www.ictvonline.org/virus taxonomy.asp, excepto para CVD-V y CVd-OS que están todavía consideradas como especies tentativas

De este conjunto de viroides, CEVd induce la enfermedad conocida como exocortis (Semancik y Weathers, 1972) y variantes específicas de HSVd causan la enfermedad conocida como caquexia (Semancik *et al*., 1988, Reanwarakorn y Semancik, 1998, 1999; Palacio-Bielsa *et al*., 2004; Serra *et al*., 2008a) (Figura 7).
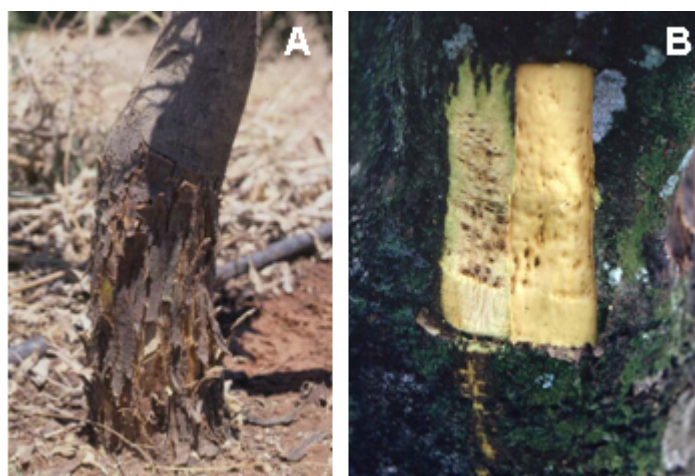
Figura. 7. (A) Síntomas de exocortis en el patrón *Poincirus trifoliata*. **(B)** Síntomas de caquexia en un madarino común injertado en naranjo amargo. (veáse que solo se observan síntomas en la variedad).

Cada uno de los viroides induce síntomas específicos en cidro Etrog, huésped experimental utilizado para el diagnóstico (Tabla 4 y Figura 8).

Tabla 4. Síntomas inducidos por los viroides de los cítricos en cidro Etrog[1]

Viroide	Síntomas						
	General		Hoja		Tallo		
	Enanismo	Epinastia	Necrosis de pecíolo	Necrosis nervio central	Lesiones necróticas	Grietas	Exudados de goma
CEVd	Acusado	Acusada	+	General	-	-	-
CBLVd	Leve	Suave	-	Puntual	Acusado	Acusado	Acusado
HSVd	-	-	+/-	-	-	-	-
CDVd	Leve	Moderada	+	General		+	Suave
CBCVd	Moderado	General	+	General	-	-	-
CVd- OS[2]	-	Puntual	+	-	-	-	-
CVd-V[3]	Leve	-	-	-	Suave	Suave	Suave

[1] Indicador cidro Etrog Arizona 861-S1 (Roistacher *et al.,* 1977).
[2] Ito *et al.* (2002).
[3] Serra *et al.* (2008).
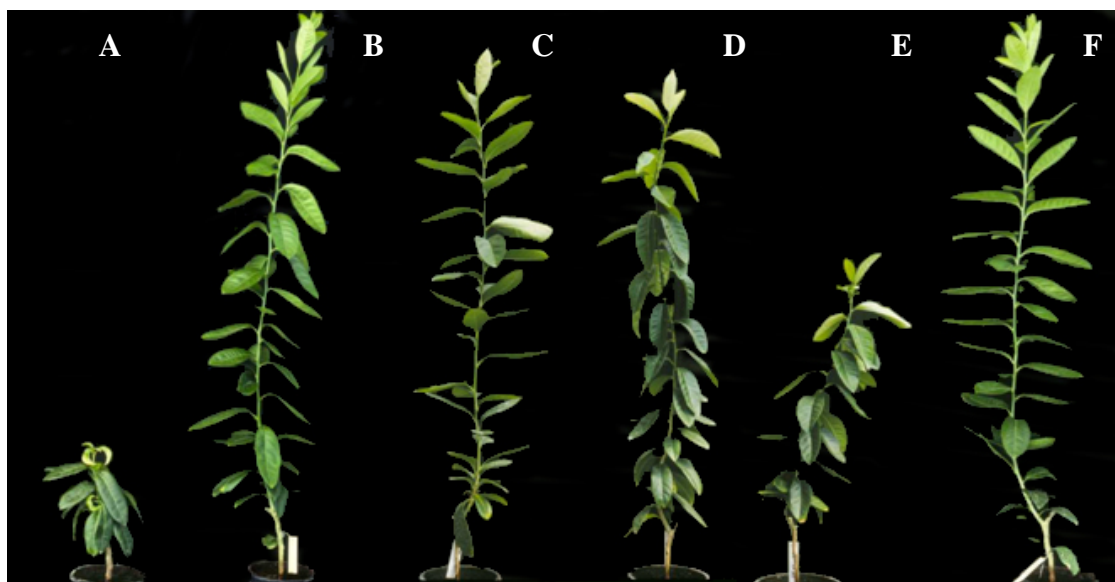
Fig. 8. Síntomas inducidos en cidro Etrog por (**A**) CEVd, (**B**) CBLVd, (**C**) HSVd, (**D**) CDVd, (**E**) CBCVd, (**F**) control sano.

El efecto enanizante que produce la infección con viroides en cítricos, se ha evaluado en distintos estudios. El objetivo es utilizar esta propiedad en beneficio del cultivo especialmente en campos establecidos con altas densidades de plantación, ya que los árboles de menor tamaño permitirían optimizar los recursos en relación con la aplicación de nutrientes y productos fitosanitarios así como para la recolección de la cosecha. Por lo tanto, ello permitiría reducir los costes de producción y mejoraría la inversión (revisado por Hutton *et al.*, 2000).

El primer trabajo dirigido a identificar factores enanizantes transmisibles por injerto fue realizado en Australia. Se evaluaron nueve factores enanizantes en naranjo Washington navel, pomelo Marsh y naranjo Valencia injertados sobre *P. trifoliata*. Después de cinco años, algunos árboles presentaban síntomas de descamaciones, mientras que otros solamente manifestaban diferentes grados de enanismo (Fraser *et al*., 1961). Posteriormente tras la identificación de CEVd como agente causal de la exocortis y de los otros viroides de cítricos (Duran-Vila *et al*., 1986; 1988), el enanismo de los aislados australianos pudo ser asociado al efecto de los viroides presentes en los aislados utilizados como factores enanizantes: CEVd, HSVd (variante tipo CVd-IIa) y CDVd (variante tipo CVd-tipo IIIa) (Gillings *et al.*, 1991). En Israel se estudió el efecto de un aislado de pomelo injertado en *P. trifoliata* que tuvo un efecto marcado sobre el tamaño de los árboles. Este aislado contenía además de CEVd, otros cuatro viroides que se diferenciaban en tamaño y que inducían diferentes grados de enanismo en cidro Etrog (Hadas *et al*., 1989).

Semancik *et al*., (1997), evaluaron los efectos enanizantes de tres aislados procedentes de diferentes regiones que contenían variantes de los viroides CBLVd, HSVd y CDVd. La infección de árboles de naranjo Valencia injertados en *P. trifoliata* con CBLVd y CDVd no afectaba el tamaño ni la producción, pero se observó una mayor producción cuando estos árboles estaban coinfectados tambien con un aislado de HSVd tipo CVd-IIa.

El único estudio donde se ha evaluado de forma sistemática el efecto de cada viroide sobre expresión de síntomas, crecimiento vegetativo (altura del árbol, diámetro del patrón, diametro de la variedad y volumen de la copa) y producción, fué realizado por Verniere *et al*. (2004). Sus resultados confirmaron los efectos enanizantes que producen las infecciones con aislados de CEVd y CDVd en clementinos injertados sobre *P. trifoliata*. El efecto resultó ser mucho menor en el caso de árboles infectados con aislados de HSVd y nulo en el caso de árboles infectados con CBLVd o CBCVd. La producción anual y la producción acumulada se vieron afectadas de acuerdo al tamaño de los árboles que manifestaban enanismo. Aunque hay informes que mencionan los beneficios que puede tener para la citricultura utilizar los viroides como agentes enanizantes, también se recomienda tener especial cuidado en estimar el costo-beneficio antes de utilizar la estrategia de infección con viroides para el control del tamaño del árbol (Verniere *et al*., 2004). Además debe tenenerse en cuenta los riesgos asociados a su posible transmisión a otros huéspedes más sensibles y/o en otras condiciones climáticas.

9.1. El viroide de la exocortis de los cítricos (CEVd).

CEVd fue identificado en 1972 como el agente causal de la exocortis (Semancik y Weathers, 1972). Es una especie del género *Pospiviroid* dentro de la familia *Pospiviroidae*. Su

tamaño oscila entre 370 y 375 nucleótidos (Gross *et al.*, 1982), forma una fuerte estructura secundaria en forma de varilla con 67% de bases apareadas en las que es posible distinguir los cinco dominios estructurales propuestos por Keese y Symons (1985).

CEVd tiene una amplia gama de huéspedes naturales y experimentales, dentro de los que se incluyen tanto especies sensibles como tolerantes. Como huéspedes naturales se cita la vid (Flores *et al.*, 1985; García-Arenal *et al.*, 1987; Rezaian *et al.*, 1988) y varias especies hortícolas como tomate, nabo, berenjena, zanahoria y haba (Mishra *et al.*, 1991, Fagoaga *et al.*, 1995; Fagoaga y Durán-Vila, 1996). A nivel experimental los ensayos de infectividad y la caracterización biológica de este viroide se ha realizado en hospederos herbáceos como *Gynura aurantiaca* (Weathers *et al.*, 1967; Chaffai *et al.*, 2007), crisantemo (*Dendranthema grandiflora*) (Visvader y Symons, 1983) y tomate (*Lycopersicum esculentum*) (Visvader y Symons, 1983). Estos hospederos desarrollan síntomas de enanismo acusado, epinastia y distorsión de las hojas.

La transmisión a huéspedes experimentales ha sido y sigue siendo una práctica común en patología vegetal. A partir de hospederos experimentales herbáceos como *G. aurantiaca* y crisantemo que manifiestan síntomas y acumulan títulos elevados de CEVd, se lograron obtener las primeras secuencias de este viroide, CEVd-C y CEVd-A, procedentes de un aislado de campo de California y otro de Australia (Gross *et al.*, 1982; Visvader *et al.*, 1982). Fue también a partir de hospederos experimentales herbáceos, y en particular el tomate, que Visvader y Symons (1985) realizaron la caracterización biológica de aislados de CEVd que permitieron relacionar motivos de secuencia con expresión de síntomas y llevaron a proponer una clasificación de las secuencias o variantes de CEVd en agresivas (Clase A) y suaves (Clase B). Estas dos clases de secuencias se diferenciaban en 26 nucleótidos que se ubicaron en las denominadas "región patogénica izquierda" (P_L) y "región patogénica derecha" (P_R), localizadas en los dominios P y V respectivamente, de la estructura secundaria del viroide (Visvader y Symons, 1985). Posteriormente utilizando *G. aurantiaca* se confirmó que efectivamente las variantes de CEVd que contenían las P_L y P_R características de la "Clase A" inducían síntomas fuertes mientras que las que contenían las P_L y P_R características de la "Clase B" inducían síntomas suaves (Chaffai *et al.*, 2007). Recientemente se han identificado variantes de CEVd en las que las secuencias de los motivos P_L y P_R no se ajustan a los definidos para la "Clase A" y la "Clase B", por lo que se ha propuesto la existencia de una nueva clase de variantes de CEVd (Bernad *et al.*, 2004b).

El papel de los dominios estructurales y en particular los motivos P_L y P_R que se encuentran en dos dominios distintos, se estudió mediante la síntesis de viroides quiméricos intraespecíficos, demostrándose que el papel modulador de la patogénesis recae en el motivo

P_L que se encuentra en el dominio P (Visvader y Symons, 1986). Tambien se han abordado estudios utilizando quimeras interespecíficas de CEVd y TASVd, concluyéndose que los dominios T_L y P afectaban la agresividad de los síntomas, mientras que los dominios V y T_R afectaban la replicación y acumulación del viroide (Sano *et al.*, 1992).

Hay que mencionar que aunque los hospederos herbáceos resultan muy útiles para llevar a cabo estudios básicos, los resultados relativos a la agresividad de distintos aislados hay que tomarlos con cautela ya que no se pueden extrapolar para predecir el efecto sobre el comportamiento de los huéspedes naturales en las condiciones del cultivo en campo. De hecho,dos aislados de CEVd caracterizados como suaves "clase B" y agresivos "clase A" en *G. aurantiaca*, indujeron un efecto similiar sobre el tamaño del árbol y la cosecha en clementinos injertados sobre *Poncirus trifoliata* (Vernière *et al.,* 2004).

Los aislados de CEVd no contienen secuencias únicas del viroide, son poblaciones más o menos heterogéneas de variantes de secuencia sobre las que el huésped parece ejercer una presión de selección. Esto se evidenció en estudios en los que después de realizar trasmisiones seriadas se recuperó una población en la que se identificaron cambios notables en la secuencia de las variantes, en el título del viroide en la planta y en las propiedades biológicas del aislado (Semancik *et al.*, 1993). Otro ejemplo que ilustra el efecto del huésped en la composición y propiedades biológicas de CEVd es el obtenido al caracterizar un aislado de este viroide recuperado de plantas de haba (*Vicia faba*) que no manifestaban síntomas y que presentaba una población muy heterogénea de variantes. Este aislado después de ser transmitido a tomate presentaba una población de variantes muy homogénea y capaz de inducir síntomas en haba (Fagoaga *et al*., 1996; Gandía *et al.*, 2007).

El efecto del huésped en la composición y estructura de poblaciones de variantes de CEVd, se ha demostrado más recientemente en cítricos al analizar naranjos trifoliados y naranjos amargos inoculados con el mismo aislado de CEVd. En los naranjos trifoliados se recuperó una población con una baja diversidad nucleotídica y una variante claramente predominante, mientras que en naranjo amargo que es un huésped asintomático, se recuperó una población con alta diversidad nucleotidíca y no se pudo identificar un haplotipo predominante (Bernad *et al*., 2004b).

9.2. El viroide del enanismo del lúpulo (HSVd).

La identificación inicial de HSVd en cítricos hacía referencia a dos RNAs con distintas movilidades electroforeticas que fueron denominados como CVd-IIa y CVd-IIb (Duran-Vila *et al.,* 1986). La posterior secuenciación permitió determinar que estos RNAs eran muy similares en tamaño, 299 (CVd-IIb) y 302 nucleótidos (CVd-IIa), y que compartían una identidad de

secuencia del 98% (Sano *et al.*, 1988; Levi y Hadidi, 1992; Hsu *et al.*, 1994). La caracterización biológica para diferenciar variantes de HSVd se evaluó inicialmente en cucurbitáceas (Shikata, 1990), en las que todas las variantes producían síntomas de enanismo y rugosidad de las hojas (Sano *et al.*, 1988) mientras que en cidro inducían síntomas muy suaves (Roistacher *et al.*, 1977). Posteriormente se demostró que variantes específicas del mismo causaban la enfermedad de la caquexia (Semancik *et al.*, 1988). La caracterización biológica y molecular mostró que los aislados que migraban en geles de poliacrilamida como el CVd-IIb eran patogénicos en cítricos e inducían la enfermedad de la caquexia, mientras que los aislados que migraban como el CVD-IIa no eran patogénicos (Semancik *et al.,* 1988). También se identificaron otras varintaes que migraban más rápidamente que las anteriores y que se denominaron CVd-IIc (Semancik y Duran- Vila, 1991).

Reanwarakorn y Semancik (1998) estudiaron la patogenicidad de CVd-IIa y CVd-IIb comparando sus secuencias, estructuras y actividad biológica en *Luffa cilindrica* y en mandarino Parson's Special. Las variantes que inducían una reacción positiva en estos huéspedes experimentales, inducían también síntomas agresivos en tangelo Orlando y *C. macrophylla* (Reanwarakorn y Semancik, 1999). Mediante la construcción de quimeras intraespecíficas entre estas dos variantes y generando mutantes artificiales se definió que la patogénesis estaba determinada por la presencia de un motivo de 6 nucleótidos localizados en el dominio V de la estructura secundaria del viroide (Reanwarakorn y Semancik, 1998). Posteriormente se confirmó la asociación del motivo conservado en el dominio V con la patogénesis y se identificó un aislado patogénico en el que dicho motivo contenía solo 5 de los 6 nucleótidos descritos anteriormente (Palacio-Bielsa *et al.*, 2004). Recientemente mediante mutaciones artificiales de HSVd (Serra *et al.,* 2008a) se confirmó que el motivo de expresión de la caquexia conformado por los 5-6 nucleótidos ubicados en el dominio V, es responsable de la inducción de los síntomas de caquexia, y que cambios mínimos dentro de esta región influyen sobre la agresividad de los síntomas e incluso pueden suprimir la expresión de los mismos.

9.3. El viroide de la hoja curvada de los cítricos (CBLVd)

El CBLVd anteriormente denominado viroide I de los cítricos y caracterizado de cidro, fue descrito inicialmente como dos RNAs (CVd-Ia y CVd Ib), con distinta migración en los análisis sPAGE (Duran-Vila *et al.,* 1988). La secuenciación demostró que se trataba de dos variantes de un nuevo viroide que pertenece al género *Apscaviroid*, dentro de la familia P*ospiviroidae.* CBLVd tiene un tamaño entre 330-340 nucleótidos y una estructura secundaria en forma de varilla. Se ha propuesto que se trata de un viroide quimérico formado por la región central de ASSVd y parte de los dominios P y T_L de CEVd. (Ashulin *et al.*, 1991). La gama de

huéspedes esta restringida casi exclusivamente a la familia de las rutáceas, aunque se ha logrado transmitir a aguacate mediante injerto heterológo (Hadas *et al*., 1992). En la especie indicadora cidro Etrog, este viroide produce necrosis puntuales en la nervadura central que causa epinastia de las hojas a veces acompañada de exudaciones de goma en el tallo y las ramas y a la pérdida de dominancia apical (Duran-Vila *et al.*, 1988).

9.4. El viroide del enanismo de los cítricos (CDVd)

La primera descripción de CDVd data del año 1988, cuando se identificaron otros RNAs que migraban como bandas distintas (CVd-IIIa, CVd-IIIb, CVd-IIIc y CVd-IIId) en análisis por sPAGE, y cuyo tamaño se estimó en un intervalo de 280 a 292 nucleótidos (Duran-Vila *et al*., 1988). La secuenciación posterior demostró que se trataba de variantes de un mismo viroide, con elevada identidad de secuencia entre ellos (Semancik y Duran-Vila, 1991; Rakowski *et al*., 1994). CVd-III recientemente nombrado como CDVd pertenece al género *Apscaviroid* dentro de la familia *Pospiviroidae*. La región TR de la estructura de varilla parece derivada de la región terminal conservada de PSTVd y de ASSVd (Stasys *et al*., 1995).

La identidad de secuencia entre las variantes CVd-IIIa y CVd-IIIb es de 95,95% con 11 cambios característicos entre ellos; mientras que la identidad de secuencia entre CVd-IIIc y CVd-IIIb es de 93,93% con 12 cambios característicos entre ellos. La secuenciación de otras fuentes de CDVd procedentes de distintos países ha demostrado que este viroide posee un genoma muy conservado (Owens *et al*., 1999) siendo las variantes similares a CVd-IIIb las más abundantes, mientras que las del tipo CVd-IIIa son poco frecuentes. Este viroide no se ha asociado con ninguna enfermedad en especies y cultivares comerciales.

CDVd tiene al cidro Etrog como único huésped experimental; esta especie indicadora es la única que manifiesta síntomas claros al ser inoculada con este viroide. Los síntomas característicos son enanismo moderado, anillado y necrosis del pecíolo que dan un aspecto de hoja caída como resultado de la curvatura del pecíolo y epinastia de hojas (Duran-Vila *et al.*, 1988).

CDVd se halla ampliamente diseminado en las regiones citrícolas del mundo, donde sus efectos pueden pasar inadvertidos ya que no produce síntomas específicos en las especies y variedades comerciales. Este viroide se ha encontrado asociado a los denominados "factores enanizantes transmisibles por injerto" en especies de interés comercial injertadas en *P. trifoliata,* y citranges Troyer y Carrizo (Gillings *et al*., 1991; Semancik *et al*., 1997; Villalobos *et al*., 1997; Owens *et al*., 2000). Por tanto, la reducción del tamaño del árbol y de la copa en cultivares de naranjo y clementino injertados sobre *P. trifoliata*, han llevado a proponerlo como agente enanizante para controlar el tamaño de los árboles, ya que su efecto en la cantidad

de cosecha puede compensarse realizando plantaciones con marcos de plantación mas densos ("high density plantings") (Roistacher *et al.*, 1993; Semancik *et al.*, 1997; Verniere *et al.*, 2004; Vidalakis *et al.*, 2004).

9.5. El viroide de la corteza agrietada de los cítricos (CBCVd).

CBCVd se identificó inicialmente como un RNA de movilidad superior a la de los otros viroides, y se le denominó viroide IV de los cítricos (CVd-IV). Es el viroide menos difundido de todos los que afectan a los cítricos. Fue inicialmente descrito en un aislado de California (Duran-Vila *et al.*, 1988) y posteriormente se identificó en aislados de Israel (Hadas *et al.*, 1989) y Turquía (Onelge *et al.*, 1996).

CBCVd tiene un tamaño de 284 nucleótidos y una estructura en forma de varilla con 71% de bases pareadas. Se propuso por el comité internacional de taxonomía de virus ICTV, como una especie del género *Cocadviroid* dentro de la familia *Pospiviroidae*. CBCVd es un viroide altamente conservado, contiene un fragmento de 80-90 nucleótidos en el dominio V y en el dominio T_R de la molécula idéntico a CEVd (Putcha *et al.*, 1991). Por esta homología con CEVd y la similitud de la región terminal izquierda con HSVd se considera como un recombinante natural. La información sobre los efectos que causa la infección con este viroide es muy limitada y se desconocía su efecto en especies y variedades comerciales. Recientemente se ha demostrado que CBCVd produce grietas en la corteza de *P. trifoliata,* pero no produce ningún efecto sobre el tamaño del árbol y la cosecha de clementinos injertados sobre este patrón (Vernière *et al.,* 2004). En la planta indicadora cidro Etrog induce enanismo, necrosis del pecíolo y epinastia.

9.6. El viroide V de los cítricos

El viroide V de los cítricos (CVd-V) fue identificado después de su transmisión a *Atalantia citroides*, una especie de un género afín a los cítricos que parece ser inmune a la infección con CEVd, CBLVd, CDVd, HSVd o CBCVd, pero que permite la replicación de CVd-V (Barbosa *et al.*, 2005). Se desconoce su origen, y probablemente pasó desapercibido en anteriores análisis por sPAGE debido a que presenta una movilidad electroforética similar a HSVd y CDVd (Barbosa *et al.*, 2005, Duran-Vila *et al.*, 1993). Después de su reciente caracterización molecular y biológica se ha propuesto como una nueva especie del genero *Apscaviroid* dentro de la familia *Pospiviroidae* (Serra *et al.*, 2008b). Este nuevo viroide tiene un genoma de 293-294 nucleótidos y contiene la CCR y la TCR típicas de los miembros del género *Apscaviroid*. Su estructura secundaria de mínima energía libre es en forma de varilla y muestra una identidad de secuencia con otros viroides inferior al 90%. Las pruebas de infectividad sobre la indicadora cidro Etrog han mostrado que induce síntomas suaves, pero al

ser coinoculado con otros miembros del genero *Apscaviroid* manifiesta una interacción sinérgica causando un enanismo muy pronunciado y síntomas en las hojas (Serra *et al*., 2008c).

A pesar de su reciente descubrimiento CVd-V se ha detectado en varias regiones productoras de cítricos como Estados Unidos, España, Nepal y el Sultanato de Omán. Tiene una amplia gama de huéspedes dentro de los cítricos, ya que se ha logrado trasmitir a naranjo dulce, mandarino, híbridos de mandarino, clementino, satsuma, limón, lima dulce Palestina, lima Tahiti, naranjo agrio, calamondin, bergamoto y kumquat (Serra *et al*., 2008c).

9.7. El viroide CVd-OS

El viroide OS de los cítricos (CVd-OS) es un viroide que se identificó en árboles de Japón, el único país en el que ha sido descrito. Posee un genoma de 330-331 nucleótidos, con la CCR característica de los miembros del genero *Apscaviroid* y una similitud de secuencia del 68% con CDVd (Ito *et al*., 2001). Se considera un viroide quimérico pues presenta una similitud de secuencia con CDVd y ADFVd en los dominios C y T_L, y con CEVd en los dominios V y T_L (Ito *et al*., 2001). Este viroide se ha detectado en variedades de naranjo dulce, en tangor (*C. reticulata* x *C. sinensis)* y en el híbrido Shiranui (*C. reticulata* x *C. sinensis*) *x C. reticulata*) únicamente en Japón (Ito *et al*., 2002). En cidro Etrog causa necrosis suave en el pecíolo y doblamiento de las hojas. Este viroide aun no ha sido considerado como una nueva especie por el ICTV debido a la escasa información sobre sus propiedades biológicas.

10. Enfermedades de los cítricos producidas por viroides

10.1. La exocortis de los cítricos

La enfermedad de la exocortis de los cítricos se describió por primera vez en California como una afección de árboles de cítricos injertados sobre *P. trifoliata*, que manifestaban descamaciones en la corteza del patrón, enanismo pronunciado del árbol y reducción de la cosecha (Fawcet y Klotz, 1948). Como se desconocía su causa, la enfermedad fua asociada a factores genéticos inherentes a las plantas de semilla de *P. trifoliata.* En la misma época se describió en Australia una enfermedad de características similares conocida como "Scaly butt" y que resultó ser transmisible por injerto, por lo que se le atribuyó una etiología viral (Bentón *et al.,* 1950). En la actualidad se conoce que estas enfermedades se deben al mismo agente causal y se las denomina exocortis.

En un principio los conocimientos sobre la biología de la enfermedad se apoyaban en las observaciones de síntomas en árboles de campo que se caracterizaban por diferentes grados de enanismo, formación de descamaciones de distinta gravedad y diferencias en el periodo necesario para la aparición de los primeros síntomas, diferencias que fueron atribuidas a las

distintas razas del agente causal (Bentón *et al.*, 1949, Calavan y Weathers, 1961; Fraser y Levvit, 1959; Salibe y Moreira, 1965a, Rodríguez *et al*., 1974). En base a este tipo de observaciones se consideró que existían distintas razas del agente causal que se clasificaron como agresivas, moderadas y suaves (Roistacher *et al.,* 1977).

Las pruebas biológicas para el diagnóstico de la exocortis en un principio se realizaban utilizando plantas de semilla de *P. trifoliata* pero la identificación del cidro como un huésped más sensible y capaz de manifestar síntomas a lo pocos meses de efectuada la inoculación llevó a abandonar el *P. trifoliata* como planta indicadora para el diagnóstico. Desde entonces se han seleccionado clones sensibles como Arizona 861 o Arizona 861-S1 y se han desarrollado protocolos para el diagnóstico biológico. Síntomas de bronceado de las puntas de las hojas, necrosis y arrugas del pecíolo, necrosis de los nervios de las hojas, epinastia y enanismo, son entre otros los síntomas observados en el clon de cidro Arizona 861-S1. Estos síntomas se consideraron en un principio como una evidencia de razas suaves, agresivas e intermedias de exocortis, pero cuando se obtuvieron preparaciones purificadas de CEVd y se inocularon en cidro los síntomas obtenidos fueron: epinastia acusada, rugosidad de las hojas y enanismo (Duran-Vila *et al*., 1988).

La transmisión de formas agresivas de la enfermedad a *Gynura aurantiaca* D.C. y a otras hospederas herbáceas (Weathers *et al*., 1967) permitió aislar y caracterizar el agente causal CEVd, un RNA infeccioso de bajo peso molecular (Semancik y Weathers, 1972). La *Gynura* utilizada como huesped experimental, mostraba síntomas característicos de enanismo, epinastia y distorsión de las hojas y permitía la producción de altos títulos del viroide (Semancik y Weathers, 1972). A partir de ese momento los trabajos de caracterización biológica y molecular de CEVd se realizaron utilizando *G. aurantiaca*, tomate y crisantemo (Flores y Semancik, 1982, Semancik y Harper, 1984; Semancik *et al*., 1973, Semancik *et al*., 1975, Visvader *et al*., 1982) y se aportaron las primeras secuencias de 371 nucleótidos de CEVd y nuevos datos sobre variantes que presentaban diferencias en el número de nucleótidos con un rango de 370 a 375 (Visvader y Symons, 1983; Visvader y Symons, 1985).

En cítricos, además de *P. trifoliata*, se conocen otras especies sensibles a exocortis, entre ellas se encuentran genotipos utilizados como portainjertos como los híbridos citrange Troyer y citrange Carrizo (*P. trifoliata* X *C. sinenesis*), la lima Rangpur (*C. limonia*), así como otras utilizadas como variedades comerciales como son la lima dulce (*C. limettioides)*, limonero (*C. limon*) y pummelo (*C. grandis*). Los síntomas en *P. trifoliata* se caracterizan por la aparición de descamaciones y grietas verticales en la corteza, amarilleo de brotes jóvenes y enanismo acusado del árbol. A pesar de que muchas especies y cultivares de cítricos son tolerantes, cuando se encuentran injertados sobre patrones sensibles pueden mostrar

amarillamiento de la copa y decaimiento general del árbol. Las pérdidas económicas que causa la enfermedad en combinaciones patrón/variedad sensibles dependen de la cepa del viroide, la edad del árbol en el momento en que tiene lugar la infección y de las condiciones climáticas en que crece el cultivo. Según los estudios desarrollados en Estados Unidos, Brasil y Australia el efecto sobre la reducción del tamaño del árbol y la producción pueden ser de hasta un 60% (Duran-Vila, 2000).

10.2. *La caquexia de los cítricos*

La xyloporosis, fue descrita por primera vez en Palestina como una enfermedad que se caracterizaba por la aparición de acanaladuras en la madera en la lima dulce de Palestina (*C. limettioides*) empleada como patrón, e inicialmente se atribuyó a causas fisiológicas (Reichert y Pelberg, 1934). Posteriormente, se describió en Florida la caquexia como una enfermedad que afectaba al tangelo Orlando y producía acanaladuras en la madera, proyecciones en la cara cambial de la corteza con fuertes impregnaciones de goma (Childs, 1950, 1952). Años más tarde, Roistacher (1988) sobre la base de una serie observaciones (transmisión mecánica, resistencia a la termoterapia y facilidad de recuperar plantas libres del patógeno mediante microinjerto de ápices calulinares) sugirió que el agente causal podía ser un viroide. Durante varios años se utilizó el término caquexia para definir la enfermedad en tangelo Orlando y se mantuvo el término xyloporosis para describir la condición descrita originalmente en limero dulce.

La caracterización de distintos aislados de campo, que inducían la enfermedad permitió aislar y purificar su agente causal al que se denominó viroide de la caquexia de los cítricos (CCaV) (Semancik *et al*., 1988). En los análisis por sPAGE, se observaron bandas de distintas movilidad electroforética que se incluyeron en el denominado grupo CVd-II de los cítricos (Duran- Vila *et al*., 1986). La caracterización biológica demostró que determinadas variantes (de movilidad similar a CVd-IIb) producían la enfermedad, mientras otras (de movilidad similar a CVd-IIa) no producían síntomas. Posteriormente se demostró que ambos tipos de variantes estaban relacionadas con el HSVd y se determinó el motivo responsable de la patogenicidad (Reanwarakorn y Semancik, 1998, Reanwarakorn y Semancik, 1999). En la planta indicadora cidro Etrog el título de este viroide era mucho mas bajo que el de otros viroides de cítricos (Duran-Vila, 2000). Tanto las variantes patogénicas como las no patogénicas de HSVd se hallan muy difundidas en todas las regiones citrícolas del mundo.

La enfermedad de la caquexia causa en especies sensibles síntomas de decoloración del floema y exudaciones de goma y punteaduras en la madera en tangelo Orlando. También afecta a otras especies sensibles como los mandarinos (*C. reticulata*), clementinos (*C.*

clementina), satsumas (*C. unshiu*), algunos mandarinos híbridos como los tangelos (*C. paradisi* x *C. tangerina*) y kumquats (*Fortunella spp*) injertados sobre cualquier patrón y en alemow (*C. macrophylla*) y lima Rangpur (*C .limonia*) dos especies empleadas como patrones.

Las pruebas biológicas en mandarino Parson's special para el diagnóstico biológico de la caquexia resultan complejas, debido al largo periodo de incubación necesario y algunas veces por la variación en la manifestación e intensidad de los síntomas (punteaduras y exudaciones de goma en la zona del injerto). Sin embargo, esta prueba sigue utilizándose para determinar la presencia del viroide causal de la caquexia en los programas de certificación.

10.3. Enfermedad de la lima Tahití

La lima Tahití (*C. lattifolia*) es una especie comercial importante y altamente valorada en países de Latinoamérica como Méjico, Brasil, Cuba y Colombia. Esta especie es muy sensible a un síndrome conocido como "quebra galho" en Brasil y que se caracteriza por la aparición de grietas longitudinales en la corteza, lo que conlleva que las ramas tiendan a quebrarse fácilmente y en las ramas gruesas pueden aparecer escamas (Salibe y Moreira 1965b, Salibe 1961; Salibe, 1978). Dado que las transmisiones a cidro Etrog indicaban que las limas Tahití eran portadoras de viroides (Salibe, 1978), se planteó la hipótesis de que el agente causal de la exocortis o algún otro viroide pudiera ser responsables del síndrome del "quebra galho". Está hipótesis estaba apoyada por la observación de que las limas Tahití afectadas de "quebra galho" e injertadas sobre lima Rangpur manifestaban síntomas de exocortis en el patrón.

En México, se han observado árboles de lima Tahití que presentan descamaciones en la corteza, en las ramas principales y en el tronco que estaban también asociados a diferentes grados de deterioro y enanismo del árbol. El análisis utilizando los métodos sPAGE y RT-PCR, confirmó la presencia de dos viroides, CEVd y HSVd, lo cual también apoyaba la hipótesis de una etiología viroidal, y concretamente CEVd, HSVd o la combinación de ambos viroides en un mismo árbol (Alvarado- Gómez *et al*., 2000).

En Cuba se ha observado también esta enfermedad que no produce daños significativos posiblemente por el uso de portainjertos tolerantes a viroides. En un estudio realizado por Ochoa *et al*., (1996) se menciona que los árboles de lima Tahití injertados sobre lima Rangpur no presentan grietas en el patrón y los síntomas de la lima se han atribuido, a otros viroides distintos a CEVd. Al evaluarse el efecto de la infección de varios aislados de viroides que contenían la combinación de CEVd y CDVd, o HSVd (variante caquexia) y CBCVd sobre el crecimiento y producción de lima Tahití injertada sobre el portainjerto *C. macropylla,* solo los árboles inoculados con el aislado que contenía la variante caquexia,

presentaron una disminución de la altura de las plantas y síntomas de clorosis, defoliación y seca de ramillas. Estos síntomas se atribuyeron a la sensibilidad del portainjerto, que presentaba punteaduras en la madera con proyecciones en la superficie interna de la corteza y acumulación de goma, síntomas típicos de caquexia (Pérez *et al.*, 2004).

Todo lo anterior sugiere que en varios países productores de lima Tahití se ha considerado la hipótesis de que la afección esté producida por algún viroide, pero la relación causa/efecto entre el CEVd u otros viroides con el síndrome del "quebra galho" no ha sido todavía demostrada.

11. Control de las enfermedades producidas por viroides

Para evitar la introducción y dispersión de las enfermedades causadas por viroides, el único método de control es de tipo preventivo. Esto solo es posible, si se han establecido programas de cuarentena, saneamiento y certificación que regulen la entrada de material del exterior y a su vez aseguren la utilización de material sano en los nuevos huertos a establecer. Para ello, es necesario disponer de métodos de detección fiables para las diferentes enfermedades causadas por virus y viroides.

Las medidas preventivas recomiendan: (i) utilizar plantas sanas procedentes de un programa de certificación para el establecimiento de nuevos huertos; (ii) utilizar yemas procedentes de varetas de plantas sanas para el injerto o sobre injerto de patrones; (iii) desinfectar las herramientas de corte y poda mediante inmersión en 1% de hipoclorito sódico (lejía comercial diluida 1:5), procedimiento que debería hacerse antes de iniciar las labores de poda o recolección de una parcela establecida con material certificado (Duran-Vila, 2000).

Estas medidas preventivas requieren de la implementación de técnicas de diagnóstico fiables que permitan monitorear la entrada de material vegetal foráneo y analizar los árboles madre que se van a utilizar como fuente de yemas. Por esta razón la constante búsqueda y mejora de métodos de detección y diagnóstico sensibles, rápidos y sencillos, son parte de los objetivos que se propone la investigación en viroides.

12. Métodos de Detección

Inicialmente la detección de viroides se realizaba por métodos biológicos, pero con el desarrollo de técnicas moleculares, se pusieron a punto otras estrategias que se están aplicando para el diagnóstico rutinario. Cualquier avance en este sentido debe basarse en la sensibilidad, fiabilidad y repetibilidad de los métodos a emplear ya que el diagnóstico erróneo de una sola planta madre conlleva la distribución de miles de plantas infectadas a los agricultores. Para una mayor fiabilidad en el diagnóstico de viroides, se recomienda utilizar al menos dos técnicas.

12.1. Métodos biológicos

Las primeras estrategias para la detección de los agentes causales de lo que hoy sabemos son enfermedades producidas por viroides, se desarrollaron antes de conocer la etiología de las mismas. En un principio el diagnóstico de la exocortis y la cachexia se hacía exclusivamente mediante pruebas biológicas, empleando plantas indicadoras seleccionadas por su sensibilidad y que se inoculaban por injerto con material procedente de las plantas a analizar. Lima Rangpur y *P. trifoliata* fueron las primeras especies que se emplearon como indicadoras para el diagnóstico biológico de exocortis, y tangelo Orlando para caquexia. Como se desconocía el periodo de incubación necesario, la evaluación de síntomas se hacía de manera empírica después de varios años de haber realizado la transmisión.

Los análisis biológicos con estas especies se abandonaron cuando se desarrollaron métodos más sensibles y rápidos utilizando otras especies indicadoras susceptibles de ser cultivadas en invernadero (Alle y Oden, 1964; Garnsey y Whidden, 1973). La sensibilidad de los métodos biológicos depende de factores como las condiciones ambientales durante las pruebas y/o la presencia de otros agentes que pueden interferir en la expresión de sintomas. Más adelante la caracterización de los agentes causales de estas enfermedades, permitieron el desarrollo de nuevos métodos de detección basados la detección del RNA viroidal.

Las limitaciones que tienen los métodos biológicos son el largo periodo de incubación necesario para la expresión de síntomas, el coste que implica el mantenimiento de invernaderos a temperaturas elevadas y los resultados a veces erráticos debidos a interferencias entre varios viroides que co-infectan una misma planta (Roistacher, 1988; Pina *et al.*, 1991).

12.1.1 Métodos biológicos para la detección de la exocortis

P. trifoliata y lima Rangpur fueron sustituidas por cidro como planta indicadora de la exocortis. Inicialmente se emplearon plantas de semilla, pero su respuesta resultó ser variable debido a que el cidro es una especie monoembriónica (Frost *et al.*, 1968). Por esta razón se empezaron a emplear propagaciones clonales de cidros que habían sido seleccionados en base a su sensibilidad, como la Arizona 861, un clon especialmente sensible y que reaccionaba de manera uniforme, y posteriormente la 861-S1 injertada sobre un patrón vigoroso como el limonero rugoso (Alle y Oden, 1964, Garnsey y Whidden, 1973, Roistacher *et al.*, 1977). Para un diagnóstico fiable se recomienda utilizar de 2 a 4 plantas de cidro por ensayo y cultivarlas en invernadero a temperaturas entre 27-32°C por un periodo de 9 a 18 meses. La observación de síntomas de enanismo, epinastia, arrugamiento de las hojas, anillamiento del pecíolo y necrosis de pecíolos, nervios y tallos en una de las plantas es suficiente para un diagnóstico

positivo (Duran-Vila, 2000). Esta prueba es considerada como el método de análisis más sensible y se ha utilizado durante muchos años.

12.1.2. Métodos biológicos para la detección de la caquexia

Para el diagnóstico de la caquexia, en los primeras ensayos se utilizaba el tangelo Orlando (Childs, 1952). Esta planta podía tardar alrededor de 4 años en exhibir los síntomas cuando se trataba del diagnóstico de aislados poco agresivos y los resultados eran erráticos y/o poco reproducibles (Roistacher, 1988). En 1973 se seleccionó como indicadora el mandarino Parson`s Special, que injertado sobre limonero rugoso o sobre otro patrón vigoroso mostraba síntomas un año después de la inoculación (Roistacher *et al.,* 1973). Para el diagnóstico fiable se recomienda utilizar de 6 a 8 plantas por ensayo que deben cultivarse en invernadero a temperaturas entre 27-32ºC por un periodo de 18 a 24 meses. La observación de acanaladuras en la madera acompañadas de exudaciones de goma por encima de la línea del injerto en al menos una de las plantas indicadoras es suficiente para un diagnóstico positivo (Roistacher *et al*., 1973). La duración de este ensayo sigue condicionando el periodo necesario para evaluar si un determinado cultivar se encuentra o no libre de patógenos. Adicionalmente hay que mencionar que cuando las plantas contienen variantes patogénicas y no patogénicas de HSVd los síntomas en el mandarino Parson`s Special pueden ser muy suaves o incluso imperceptibles debido a fenómenos de interferencia entre ambas variantes que dificultan o impiden la expresión de los síntomas (Pina *et al*., 1991).

12.1.3. Métodos biológicos para la detección de otros viroides

La caracterización biológica de los viroides demostró que todos causaban síntomas específicos en la selección 861-S1 de cidro Etrog. Aunque inicialmente este genotipo había sido seleccionado para el diagnóstico de la exocortis, en realidad manifestaba síntomas al ser inoculado con cualquiera de los viroides de cítricos. Por tanto el cidro Etrog debe considerarse como un indicador general de viroides, que aunque muy sensible no resulta específico para el diagnóstico de plantas co-infectadas con varios de ellos, una situación muy frecuente en el caso de aislados de campo que comúnmente contienen una mezcla de varios viroides. Por lo tanto las pruebas de infectividad utilizando el cidro Etrog como planta indicadora permiten diagnosticar si una planta está infectada o no con viroides, pero no determinar con qué viroides se halla infectada (Duran-Vila, 2000).

El diagnóstico biológico tiene algunas desventajas, y entre ellas cabe mencionar: (1) que no es un método práctico cuando se precisa hacer diagnósticos a gran escala; (2) que se requiere espacio para producir y mantener las plantas indicadoras durante mucho tiempo; (3) los elevados costes para mantener los invernaderos y las plantas indicadoras a temperaturas

adecuadas (28 a 32 ºC); (4) el largo periodo de tiempo necesario para evaluar los síntomas de algunos viroides; y (5) que se requiere operadores expertos para evaluar correctamente los síntomas. Aun con estas limitaciones, el método biológico es fiable y continúa utilizándose tanto en programas de saneamiento y cuarentena como para pruebas de infectividad en estudios básicos de viroides.

12.2. Métodos moleculares

Los métodos moleculares consisten en el análisis de los ácidos nucleicos mediante: a) análisis de ácidos nucleicos por electroforesis secuencial en geles de poliacrilamida (sPAGE), b) hibridación molecular, c) retrotranscripción y amplificación por PCR (RT-PCR).

La utilización de uno u otro de estos métodos depende de los propósitos del diagnostico, de la disponibilidad de personal entrenado y del título del viroide en el tejido infectado. La RT-PCR se ha intentado adoptar como una técnica de diagnóstico rutinario por su alta sensibilidad y por la posibilidad de realizar el diagnóstico a partir de especies y variedades comerciales, incluso cuando el viroide se encuentra en títulos muy bajos. Sin embargo esta sensibilidad está condicionada por la frecuencia con la que se dan falsos positivos debido a contaminaciones con amplicones, e incluso falsos negativos, por lo que algunos expertos propugnan la hibridación como uno de los métodos más fiables para el diagnóstico (Mülbach *et al*., 2003).

12.2.1. Extracción de ácidos nucleicos y análisis por electroforesis

La electroforesis permite la separación de los ácidos nucleicos mediante su migración en un gel de porosidad adecuada sometido a un campo eléctrico. En los primeros estudios realizados para identificar los agentes causales de la enfermedad del tubérculo fusiforme de la patata y de la exocortis de los cítricos (Diener, 1971; Semancik y Weathers, 1972) se utilizó la electroforesis en geles de poliacrilamida (PAGE). Con ello se demostró que el peso molecular de los RNAs implicados era inferior al de los virus (Hanold *et al.,* 2003). Posteriormente, se desarrollaron protocolos de doble electroforesis en los que las preparaciones de ácidos nucleicos se sometían a una electroforesis en condiciones no desnaturalizantes seguida de una segunda electroforesis en condiciones desnaturalizantes. Entre ellos se menciona el PAGE bidimensional (Schumacher *et al*., 1983) y el "return" PAGE, descritos inicialmente para detección de otros viroides (Schumacher *et al*., 1986; Singh y Boucher, 1987) y posteriormente ensayados para viroides de cítricos (Duran-Vila *et al*., 1991), y el PAGE secuencial (sPAGE) (Semancik y Harper, 1984). El sPAGE y la tinción de los ácidos nucleicos con nitrato de plata, resultó un método satisfactorio para detectar todos los viroides de cítricos a partir de cidros inoculados (Duran-Vila *et al*., 1993). Hay que mencionar que el sPAGE es solo fiable para la

detección de viroides en cidro Etrog donde se acumulan a concentraciones suficientemente elevadas para poder ser detectados, incluso antes de la manifestación de síntomas. El análisis por sPAGE, es un método adecuado para el descubrimiento de nuevos viroides y se está utilizando en programas de cuarentena y certificación y es junto con el bioensayo en cidro la técnica estándar de detección.

12.2.2. Métodos de hibridación molecular

El diagnóstico por hibridación molecular consiste en someter las preparaciones de ácidos nucleicos a condiciones que permitan la unión (hibridación) del viroide a detectar con una sonda específica. Normalmente la sonda consiste en RNAs o DNAs complementarios a la secuencia del viroide a analizar y deben contener nucleótidos radioactivos o moléculas de fácil detección por métodos colorimétricos o quimioluminiscentes. La técnica requiere de pasos previos como la síntesis del DNA o RNA complementario a la secuencia del viroide con el correspondiente marcaje radiactivo o químico y de la transferencia de las preparaciones de ácidos nucleicos a una membrana con carga positiva, antes de realizar la hibridación. El potencial de esta técnica se demostró cuando se utilizó para la detección de PSTVd (Owens y Diener, 1981), y a partir de ese momento se diseñaron distintas estrategias de hibridación molecular como la hibridación *Dot blot*, la hibridación *northern* y la hibridación de improntas de tejido (Mülbach *et al.,* 2003). La sensibilidad y fiabilidad de estos métodos depende de la concentración y distribución de los viroides en la planta, de la recuperación del viroide durante el proceso de extracción y de la calidad de las sondas (Romero-Durbán *et al.*, 1995).

La hibridación de improntas permite alcanzar resultados con una mínima manipulación de las muestras. Esto se consigue aplicando a la membrana improntas de secciones de tallo, hojas o frutos de forma que la savia del tejido (y los viroides) queda depositada en la membrana con lo que se evita la extracción de los ácidos nucleicos de la muestra. Se puede utilizar para el diagnóstico de rutina ya que permite procesar un gran número de muestras con rapidez, y ofrece la posibilidad de preparar las membranas en el campo o en el invernadero (Romero-Durban *et al.,* 1995; Mulbach *et al.,* 2003). Utilizando esta técnica se logró la detección de CEVd en cidro, crisantemo, pepino, gynura y tomate, HSVd en cidro, pepino, melocotonero y tomate, CSVd en crisantemo, pero no ASBVd en aguacate (Romero-Durbán *et al.,* 1995). La sensibilidad, al igual que con otros métodos de detección, está condicionada por las características del viroide a detectar y el título que presenta en el tejido a analizar.

La aplicación de este método para el diagnóstico de viroides en cítricos mostró que solo se alcanzaba la sensibilidad adecuada a partir de tejidos de cidro Etrog y se propuso su utilización como complemento al diagnóstico biológico (Palacio-Bielsa *et al*., 1999). Se

estandarizó el método de forma que utilizando 20 ng de sondas de DNA marcadas con digoxigenina (DIG) por cm^2 de membrana, se obtenían señales de hibridación adecuadas. El método permite detectar todos los viroides descritos en cítricos individualmente o en un ensayo único utilizando una mezcla de sondas con una sensibilidad comparable a la alcanzada mediante sPAGE. Por otra parte, las sondas de DNA sintetizadas por PCR resultaron ser muy estables, susceptibles de ser almacenadas durante largos periodos de tiempo, de fácil manipulación y con una sensibilidad similar a las sondas de cRNA (Palacio *et al*., 2000). La especificidad del método permite identificar los viroides de una muestra a nivel de especie, pero no discriminar variantes dentro de una especie. Por ejemplo en el caso de las variantes de HSVd en cítricos, el método no permite discriminar variantes patogénicas de las no patogénicas (Duran-Vila, 2000).

La hibridación por *Dot-blot* (en gotas), consiste en la aplicación directa de preparaciones de ácidos nucleicos en un soporte sólido, como son las membranas de nitrocelulosa o de nylon, que una vez fijadas pueden hibridarse con las sondas correspondientes. Este método es más sensible y menos errático que la hibridación de improntas pero conlleva el proceso de extracción de los ácidos nucleicos. Esta técnica es utilizada de forma rutinaria para el análisis de viroides en programas de diagnóstico. La hibridación *Dot-blot*, con sondas de cDNA marcadas radiactivamente se ha utilizado para detectar CEVd en cidro (Flores *et al*., 1988) y en distintas especies de cítricos cultivadas en campo (Gillings *et al*., 1988; Broadbent *et al*., 1988), así como en plantas infectadas con CEVd, HSVd o con infecciones múltiples de viroides (Cohen *et al*., 2006). Sin embargo los intentos llevados a cabo en nuestro laboratorio para implementar esta técnica en el diagnóstico rutinario a partir de especies y variedades comerciales, no dieron resultados satisfactorios ya que el ruido de fondo debido a hibridaciones inespecíficas no permitía discriminar entre plantas libres de viroides y plantas portadoras de bajos títulos del viroide.

La hibridación en formato *northern-blot* es un método por el que los ácidos ribonucleicos son transferidos a la membrana después de haber sido sometidos a electroforesis y por tanto haberse separado como bandas discretas en el gel. Los ácidos ribonucleicos del gel o de una porción del mismo se transfieren a la membrana conservando el mismo perfil de la electroforesis, y posteriormente se hibridan con sondas específicas. Este método es muy sensible y fiable porque permite identificar la molécula del viroide en una región concreta del gel evitando el riesgo de falsos positivos que se observa en el caso de la hibridación *Dot-blot*. El *northen-blot* ha sido utilizado fundamentalmente en estudios básicos de investigación y ha permitido identificar y discriminar las moléculas de polaridad positiva y negativa y establecer el concepto de replicación de los viroides a través del mecanismo del círculo rodante (Branch y

Robertson, 1984; Daròs, *et al.,* 1994). También se ha utilizado con éxito en investigaciones fitopatológicas, para discriminar viroides con alta similitud de secuencia como ASSVd y ADFVd (Di Serio *et al.*, 2001), para estimar similitudes de secuencia entre viroides de distinta movilidad electroforética (Duran-Vila *et al.*, 1988; Semancik, 1988), o para detectar CEVd y HSVd a partir de especies cultivadas en campo utilizando tanto sondas específicas como multisondas (Albanese *et al*., 1991; Cohen *et al.*, 2006).

12.2.3. Retrotranscripción y amplificación (RT-PCR).

La reacción en cadena de la polimerasa (*Polimerase Chain Reaction*, PCR) es una técnica que permite incrementar *in vitro* secuencias específicas de DNA aun cuando se encuentren en concentraciones muy bajas La introducción de un paso de retrotranscripción antes de la amplificación por PCR, ha hecho posible su aplicación en el estudio de patógenos con genomas de RNA como virus y viroides

Dada su gran sensibilidad, esta técnica se ha intentado adaptar para el diagnóstico rutinario de patógenos, no obstante su propia sensibilidad conlleva problemas asociados a la amplificación de amplicones contaminantes lo que dificulta diferenciar infecciones reales de falsos positivos. Otros aspectos limitantes que cabe destacar son el efecto de determinados compuestos de la planta que pueden actuar como inhibidores de las reacciones como son los polifenoles, polisacáridos y ribonucleasas endógenas que generan falsos negativos en las reacciones RT-PCR (Gibb y Padovan, 1994; Singh *et al*., 2002).

Su utilización para la detección de viroides se encuentra también limitada por la síntesis poco eficiente de fragmentos suficientemente largos de cDNA durante la retrotranscipción (Bernad y Duran-Vila, 2006). Como prerrequisitos para el uso de la RT-PCR en la detección de viroides se ha mencionado la necesidad de utilizar preparaciones altamente purificadas, la optimización de las condiciones para la producción de fragmentos largos de cDNA y una cuidadosa selección de cebadores (Ragozzino *et al.,* 2004). En este sentido, se desarrolló un protocolo de RT-PCR basado en la síntesis del cDNA del viroide por retrotrascripción a 60°C utilizando un cebador largo de 27 nucleótidos y una transcriptasa reversa termoestable, lo que seguido de la síntesis de una segunda hebra y amplificación por PCR dió resultados satisfactorios. Esta técnica, se validó ensayando preparaciones obtenidas mediante distintos métodos de extracción de ácidos nucleicos y a partir de distintas especies hospedadoras de viroides (Bernad y Duran-Vila, 2006). Con la disponibilidad de esta técnica, se ha logrado mejorar la detección y caracterización de todos los viroides de cítricos, y discriminar entre variantes suaves y agresivas de CEVd, y entre variantes patogénicas y no patogénicas de HSVd (Bernad y Duran-Vila, 2006).

La RT-PCR se está utilizando en algunos países como método de detección para confirmar la infección de árboles de campo con distintos viroides (Sieburth *et al*., 2002), e incluso se recomienda como un substituto de los métodos de detección biológica en los programas de saneamiento, monitoreo de árboles madres en bloques de fundación y para el programa de producción de yemas libres de viroides (Sieburth *et al*., 2002, Madhurababu *et al*., 2007). No obstante hay que mencionar que este método aun presenta serios inconvenientes relacionados con la obtención de falsos positivos o falsos negativos.

12.3 Combinación de métodos biológicos y moleculares

La detección de viroides es imprescindible en los programas de cuarentena, saneamiento y certificación, y antes de iniciar propagaciones comerciales. Las técnicas biológicas y moleculares anteriormente descritas tienen ventajas e inconvenientes. En este sentido, la combinación de métodos biológicos y moleculares ha dado resultados muy satisfactorios tanto para un diagnóstico fiable como para la identificación de nuevos viroides.

El análisis biológico en invernadero permite el diagnóstico de todos los viroides sobre la base de la reacción que causan en cidro Etrog, mientras que con los métodos moleculares se pueden confirmar dichos resultados y alcanzar una mayor especificidad. Para el diagnóstico de viroides de cítricos, se ha propuesto la utilización de métodos mixtos de análisis que combinan las propiedades de los métodos biológicos y de los moleculares. Estos métodos mixtos permiten acortar el periodo necesario para un diagnóstico fiable y son más sensibles y específicos que los métodos biológicos convencionales. Después de demostrar que todos los viroides se replican y acumulan en cidro Etrog a títulos detectables, incluso cuando las condiciones de incubación no son las óptimas para la expresión de los síntomas, se ha propuesto el empleo del cidro Etrog para obtener una amplificación biológica, seguido de un análisis por sPAGE o hibridación molecular después de un periodo de incubación de entre 3 y 6 meses (Duran-Vila *et al*., 1993).

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Objetivos

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La utilización de técnicas moleculares ha conllevado notables avances en la caracterización de los viroides como agentes patógenos de especies vegetales cultivadas. Las medidas de control de estos patógenos son de tipo preventivo y se basan en la disponibilidad de material de propagación sano, producido a través de programas de cuarentena, saneamiento y certificación. Para ello es imprescindible disponer de métodos de detección sensibles y fiables. En cítricos la detección se realiza fundamentalmente mediante métodos biológicos que se habían desarrollado para el diagnóstico de exocortis y cachexia antes de que se hubieran descubierto sus agentes causales. En la actualidad el diagnóstico se realiza utilizando también métodos moleculares que complementan a los biológicos, y que consisten en la inoculación previa en cidro Etrog (*Citrus medica*) que actúa como un bioamplificador, en el que todos los viroides descritos son detectados de forma específica por métodos moleculares. Esta estrategia es sensible, fiable pero tiene un elevado coste, por lo que el diseño de estrategias moleculares que permitan realizar el diagnóstico a partir de especies y variedades cultivadas en condiciones de campo sigue siendo un reto.

En el primer capítulo se aborda el desarrollo de un método de detección basado en la hibridación *northern* utilizando sondas marcadas con digoxigenina. Los objetivos del primer capítulo son:

1. Diseñar y optimizar un protocolo de hibridación northern que permita detectar *Citrus exocortis viroid* (CEVd) en árboles de naranjo Washigton navel (*C. sinensis*) injertados sobre citrange Carrizo (*Poncirus trifoliata* X *C. sinensis*).

2. Validar el protocolo para la detección de otros viroides descritos en cítricos en árboles de naranjo Washington navel injertados sobre citrange Carrizo, y a partir de muestras recolectadas en distintas épocas del año.

3. Comprobar la sensibilidad del protocolo para la detección de viroides en otras especies y variedades de cítricos.

Con la disponibilidad de esta técnica se han podido realizar prospecciones en cítricos cultivados en distintas regiones citrícolas de Colombia, Perú y Brasil, como estrategia básica para desarrollar el trabajo que se presenta en los capítulos segundo y tercero. Los objetivos del segundo capítulo son:

4. Determinar la infección con viroides en muestras recolectadas en distintas regiones citrícolas de Colombia y en el Banco de Germoplasma de Palmira.

5. Caracterizar molecularmente las especies de viroides identificadas, CEVd, *Hop stunt viroid* (HSVd) y *Citrus dwarfing viroid* (CDVd).

6. Caracterizar un aislado de CEVd procedente de un cidro Etrog de Palmira que no manifestaba síntomas y obtener mutantes que permitan identificar los cambios responsables de su inesperado comportamiento biológico.

Las prospecciones en Perú y Brasil se realizan en árboles de lima Tahití (*C. latifolia*) que manifestaban el síndrome conocido en Brasil como "quebra galho". Los objetivos del tercer capítulo son:

7. Identificar y caracterizar molecularmente los viroides en árboles de lima Tahití que manifestaban el síndrome del "quebra galho".

8. Determinar que viroide causa el síndrome del "quebra galho" mediante el seguimiento de una parcela experimental establecida con árboles de lima Tahití que habían sido inoculados con distintos viroides.

El CDVd se encuentra muy difundido en diferentes regiones citrícolas pero sus efectos pasan inadvertidos debido a que no produce síntomas específicos aunque se ha probado que causa una reducción del tamaño del árbol. En el capítulo cuarto se ha abordado la identificación y caracterización biológica de variantes de CDVd. Los objetivos del cuarto capítulo son:

9. Caracterizar 33 aislados de CDVd e identificar la/s variante/s de secuencia mas frecuentes de cada aislado, e inferir las relaciones filogenéticas entre las variantes identificadas y las de referencia.

10. Seleccionar las variantes representativas de los distintos grupos filogenéticos para su caracterización biológica en cidro Etrog y establecer la relación entre las diferencias nucleotídicas características de las variantes caracterizadas y la modulación de los síntomas inducidos.

La disponibilidad de una parcela experimental de naranjo dulce Washington navel injertado en citrange Carrizo e inoculada con distintas fuentes de viroides ha permitido a lo largo de cuatro años tomar datos sobre su comportamiento agronómico cuyo análisis se aborda en el capítulo quinto. Los objetivos del capítulo quinto son:

11. Analizar los parámetros de crecimiento, cosecha y calidad de la fruta de árboles sanos e infectados con distintos viroides.

12. Identificar los síntomas inducidos por los distintos viroides.

Capítulo I

A novel hybridization approach for detection of citrus viroids

N. Murcia, P. Serra, A. Olmos, N. Duran-Vila

Abstract

Citrus plants are natural hosts of several viroid species all belonging to the family *Pospiviroidae.* Previous attempts to detect viroids from field-grown species and cultivars yielded erratic results unless analyses were performed using Etrog citron as secondary bio-amplification host. To overcome the use of Etrog citron a number of RT-PCR approaches have been proposed with different degrees of success. Here we report the suitability of an easy to handle northern hybridization protocol for viroid detection of samples collected from field grown citrus species and cultivars. The protocol involves: (i) nucleic acid preparations from bark tissue samples collected from field grown trees regardless of the growing season and storage conditions; (ii) separation in 5% PAGE or 1% agarose, blotting to membrane and fixing; (iii) hybridization with viroid specific DIG-labelled probes and detection with anti-DIG-alkaline phosphatase conjugate and autoradiography with the CSPD substrate. The method has been tested with viroid infected trees of sweet orange, lemon, mandarin, grapefruit, sour orange, Swingle citrumelo, Tahiti lime and Mexican lime. This novel hybridization approach is extremely sensitive, easy to handle and shortens the time needed for reliable viroid indexing tests. The suitability of PCR generated DIG-labelled probes and the sensitivity achieved when the samples are separated and blotted from non-denaturing gels are discussed.

Introduction

Viroids are small (246-401 nucleotides), covalently closed, single stranded RNAs that infect and replicate in their host plants, which may develop disease symptoms or simply act as symptomless carriers. Viroids are classified into two families, *Pospiviroidae,* composed of species with a Central Conserved Region (CCR) and without hammerhead ribozymes, and *Avsunviroidae,* composed of members lacking CCR but able to self-cleave in both polarity strands through hammerhead ribozymes (Flores *et al.*, 2005).

Citrus trees are natural hosts of several viroids, all of which belong to the *Pospiviroidae* family. *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), recently renamed *Citrus dwarfing viroid* (CDVd), and *Citrus viroid IV* (CVd-IV), recently renamed *Citrus bark cracking viroid* (CBCVd) (Duran-Vila *et al.*, 1986,1988) are included in the present classification scheme proposed by International Committee on Taxonomy of Viruses (ICTV), whereas Citrus viroid original sample (CVd-OS) (Ito *et al.*, 2001) and Citrus viroid V (CVd-V) (Serra *et al.,* 2008) are still tentative new species. CEVd and HSVd are the causal agents of exocortis and cachexia, respectively (Semancik *et al.*, 1972; 1997). Although CBLVd and CDVd do not induce specific disease symptoms, they can cause reduction of tree size and fruit crop

(Semancik *et al*., 1997; Hutton *et al.*, 2000; Vidalakis *et al.*, 2004; Vernière *et al.*, 2004). CBCVd, which appears to be less widespread than other viroids, causes severe bark cracking on sensitive species (Vernière *et al.*, 2004).

Diagnosis of exocortis and cachexia diseases was initially performed by biological indexing using, respectively Etrog citron (*Citrus medica* L.) (Calavan, 1968) and Parson's Special mandarin (Roistacher *et al.*, 1973) as the indicator plants. Attempts to use electrophoresis or molecular hybridization analysis resulted in unreliable results because in many citrus species and cultivars, viroids do not accumulate at high enough titers to be detected (Palacio *et al.,* 2000)**.** However, since all citrus viroids do accumulate at detectable titers in Etrog citron, this species has been used as a secondary bio-amplification host, which, when coupled with molecular analysis, is a very sensitive and specific procedure for indexing purposes (Palacio *et al.,* 2000; Duran-Vila *et al*., 1993).

More recently and because of its great sensitivity, RT-PCR is considered as a desirable alternative to other diagnostic methods and many protocols have been proposed (Yang *et al.,* 1992; Nakajara *et al*., 1999; Ito *et al.*, 2002; Ragozzino *et al.,* 2004*;* Reanwarakorn and Semancik, 1999; Bernad and Duran-Vila, 2006; Tessitori *et al*., 2004). Unfortunately, the experience accumulated over the years has shown that false positives due to amplicon contamination and false negatives due to the failure to generate a cDNA of suitable size during reverse transcription are not infrequent (Bernad and Duran-Vila, 2006). As a consequence, it has been recommended that at least two diagnostic methods should be used for viroid identification purposes.

The objective of the present work was to define a molecular hybridization strategy for efficient and consistent detection of citrus viroids without the need of using Etrog citron as a secondary bio-amplification host. The approach involves (i) the design and optimization of a molecular hybridization protocol for detection of CEVd; (ii) the assessment of the protocol for detection of other citrus viroids; and (iii) test the sensitivity of the protocol for viroid detection in several citrus species and cultivars.

Materials and methods

Plant materials and viroid sources

Unless otherwise stated, viroid infected 'Washington navel' sweet orange (*C. sinensis* (L.) Osb.) trees growing in a field plot at the Instituto Valenciano de Investigaciones Agrarias (IVIA) were used as a source of tissue. Viroid-infected Etrog citron plants maintained in a

greenhouse at 28-32ºC were used as positive controls. Non-inoculated 'Washington navel' sweet orange and Etrog citron trees were used as negative controls.

The viroid sources used to inoculate the 'Washington navel' sweet orange were CEVd (isolate CEVd-117) (Gandía *et al.*, 2005), HSVd (isolates IIa-117 and X-707) (Palacio-Bielsa *et al.*, 2005), CBLVd (isolate CVd-Ia) (Foissac and Duran-Vila, 2000), CDVd (isolate CVd-IIIb) (Foissac and Duran-Vila, 2000) and CBCVd (Francis *et al.*, 1995). Whenever possible, and according to the availability of viroid infected plants, assays were conducted with different hosts and different field isolates of the same viroid to verify the suitability of the technique.

Extraction methods

Bark (5g) stripped from young shoots was reduced to powder in liquid nitrogen and homogenized in 5 ml of extraction buffer (0.4 M Tris-HCl pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7.0; 4% (v/v) 2-mercaptoethanol) and 15 ml of water-saturated phenol. The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 µl of TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM MgCl$_2$ pH 7.4) (Semancik, *et al.,* 1975). Aliquots of these nucleic acid preparations were used for polyacrylamide gel electrophoreses (PAGE), sequential PAGE (sPAGE) and *northern blot* hybridization analysis.

RNA analysis by PAGE and sPAGE

Aliquots (20 µl equivalent to 300 mg of fresh weight tissue) of the nucleic acid preparations were subjected to 5% PAGE under non-denaturing conditions (2 h, 60 mA) and stained with ethidium bromide (Morris and Wright, 1975). For sPAGE analysis, a segment of the ethidium bromide stained gel, containing CEVd and 7S RNA was subjected to a second PAGE containing 8M urea, at 18 mA for 4 hours (Rivera-Bustamante *et al.,* 1986). Unless otherwise stated, viroid bands were visualized by silver staining (Igloi, 1983).

Northern blot hybridization

The RNAs separated by PAGE or sPAGE were electroblotted (400 mA for 1 h) from the gel to positively charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) and immobilized by UV cross-linking. Unless otherwise stated, DIG-labeled viroid-specific probes were synthesized by PCR using as templates plasmids containing full-length viroid DNA inserts as described by Palacio-Bielsa *et al.,* (2000)**.** The sequences of the cloned inserts are available in the GenBank: CEVd (AJ54795), HSVd (AF213483), CBLVd (AF040721), CDVd (EU93401) and CBCVd (X14638). The primers used correspond to the two ends of the cloned inserts: CEVd (CEVd-

R1, CEVd-F1), HSVd (HSVd-R1, HSVd-F1), CBLVd (CBLVd-R1, CBLVd-F1), CDVd (CVd-III-R1, CVd-III-F1) and CBCVd (CVd-IV-R1, CVd-IV-F1) as described by Bernad and Duran-Vila (2006).

Prehybridization and hybridization were performed in 50% formamide and 5×SSC buffer (SSC: 150 mM NaCl; 15 mM sodium citrate; pH 7.0) containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking solution (Roche). After hybridization the membranes were washed twice in 2×SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1×SSC, 0.1% SDS at 60ºC for 60 min. The DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualized with the chemiluminiscence substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo $[3.3.1.1^{3,7}]$ decan}-4-yl) phenyl phosphate (CSPD) (Roche), and revealed by autoradiography

Probe quantification by Real time PCR

After northern hybridization with DIG-labeled DNA probes, the probe bound to the target viroid was recovered from the hybridized membrane by transferring the corresponding portions of the membranes into tubes containing 100 μl of buffer A (0.1 M Glycine, including 0.05 M NaCl) that were vortexed vigorously and placed on ice (Osman and Rowhani , 2006)

The CEVd variant V1 previously cloned in the pT7-Blue vector (Novagen) (Gandía *et al.,* 2005) was used as the control to generate the standard curves. The amount of DNA (μg) was quantified by UV densitometry. Conversion of microgram of DNA to picomole was performed considering the average molecular weight of a deoxinucleotide (330 Da) and the number of bases of the plasmid (3375bp). The following mathematical formula was applied: pmol of DNA = μg (of DNA) x (10^6 pg/1 μg) x (1 pmol/660 pg) x (1/3375bp). Avogadro constant (Avogadro, 1811) was used to estimate the number of copies (6.023 x 10^{23} molecules/mol). The number of molecules per μl was 2.4 x 10^{10}. Ten-fold serial dilutions were prepared, aliquoted and stored at −80ºC until use. Dilutions from 2.4 x 10^8 to 2.4 were employed to generate the standard curve.

StepOne Plus (Applied Biosystems) was used to perform real time PCR. The amplification mixture for each reaction contained 1X SYBR Green Master Mix (Applied Biosystems), 10 U RNase Inhibitor (Applied Biosystems), 6.25 U MultiScribe reverse transcriptase (Applied Biosystems), 300 nM primer CEVd-R1 and 300 nM primer CEVd-F1 (Bernad and Duran-Vila, 2006). PCR protocol was as follows: 40 cycles of amplification (94ºC for 30 s, 60ºC for 45s). A dissociation step from 60ºC to 95ºC was added in all cases, to analyze the specificity of the reactions. A standard curve was performed in each assay using

serial dilutions of the control. Data acquisition and analysis were performed with the OneStep Plus software. The default threshold set by the machine was slightly adjusted above the noise to the linear part of the growth curve, at its narrowest point according to the manufacturers.

Results and discussion

Detection of CEVd in field grown sweet orange trees

When nucleic acid preparations from Etrog citron and 'Washington navel' sweet orange trees were subjected to PAGE and ethidium bromide staining, CEVd was detectable only from Etrog citron, a host that accumulates high viroid titers (Fig. 1A). When a segment of the ethidium bromide stained gel containing CEVd and 7S RNA was subjected to a second PAGE (in the presence of 8 M urea) and silver staining, a procedure known as sPAGE and commonly used for viroid analysis, faint bands with the characteristic mobility of the circular form of CEVd were observed in the samples from infected sweet orange trees (Fig. 1B, lanes 1 and 2).

Detection more sensitive than that achieved with silver staining was obtained when the RNAs were transferred from the gel to a membrane and submitted to northern hybridization with a CEVd-specific probe. Under these conditions, not only the circular form (CEVd-c) but also the linear (CEVd-l) form of the viroid molecule was visible (Fig. 1C). Both sPAGE and hybridization confirmed that in contrast to the samples from Etrog citron, the CEVd titers in sweet orange were rather low.

Unexpectedly, when the RNAs were transferred from the non-denaturing gel onto a membrane and hybridized under the same conditions than above, CEVd was easily detected in all the samples from infected trees, including those from sweet orange (Fig. 1D).

Under the conditions of Fig. 1D, CEVd-c and CEVd-l moved as a single band with the mobility characteristic of the rod-like secondary structure of CEVd. Even though northern hybridization for viroid detection is normally performed on RNAs transferred to a membrane from urea-containing gels where the viroid molecules are already denatured, the results obtained by transferring the RNAs from a single non-denaturing gel suggest that the presence of formamide in the hybridization solution was probably sufficient to denature the viroid molecule, which, otherwise, would not have bound to the probe.
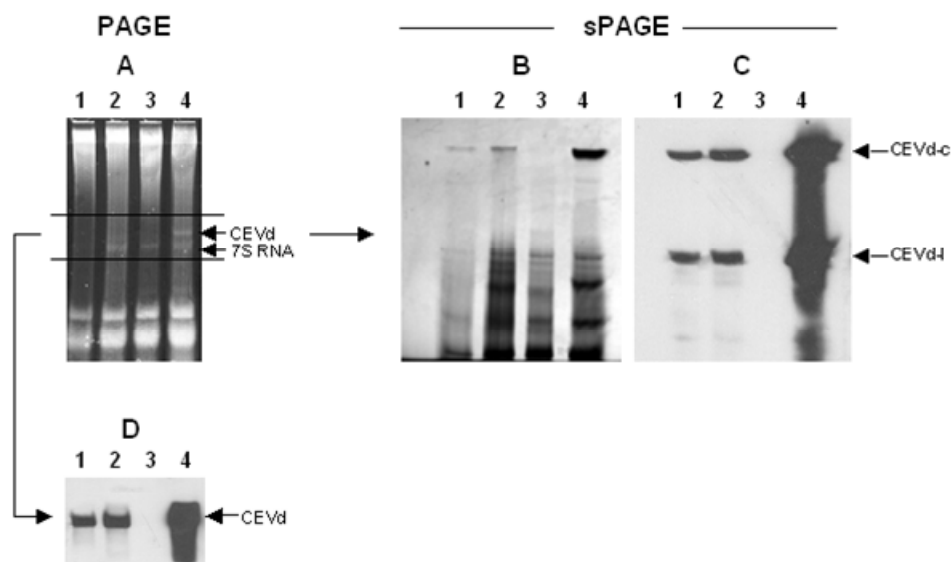
Fig. 1. Analysis of 'Washington navel' sweet orange and Etrog citron infected with CEVd. (A) Samples subjected to 5% PAGE and ethidium bromide staining. (B) A segment of the gel was excised (see lines in A) and subjected to a second 5% PAGE in a urea containing gel (sPAGE) and silver staining. (C) Samples subjected to sPAGE, electrotransferred to a Nylon membrane and hybridized with a DIG-labelled CEVd probe. (D) Samples subjected to 5% PAGE, electrotransferred to a Nylon membrane and hybridized with a DIG-labelled CEVd probe. Samples are: (1, 2) Infected 'Washington navel' sweet orange; (3) Non-infected 'Washington navel' sweet orange; (4) Infected Etrog citron. CEVd-c and CEVd-l show the circular and linear forms of CEVd.

As illustrated in Fig. 2 (lane 4), CEVd can be readily detected in sweet orange even though its titer is about hundred times lower than that in Etrog citron. The great sensitivity of northern hybridization for the detection of CEVd in RNA samples resulting from a single, non-denaturing PAGE, rather than a first PAGE followed by the denaturating sPAGE, suggested that this strategy could probably be used as a general detection procedure, not only for CEVd, but for all viroids, even when present in low titers in commercial citrus cultivars, rather than Etrog citron indicator plants. This approach is very sensitive, easy to handle and provides the desired indexing results in less than a week.

Viroid-probe binding properties

Since the DIG-labelled probe used in the northern hybridization assays described above was generated by PCR, it contained equimolar amounts of DIG-cDNA and DIG-hDNA. Given the self-complementarity of the CEVd molecule, both DNA strands of the probe (DIG-cDNA plus DIG-hDNA) could theoretically bind to the viroid and/or even bind to each other. Individual DIG-cDNA and DIG-hDNA probes were synthesized by unidirectional PCR using as templates two cloned, full-length CEVd-cDNAs inserted in opposite directions within the pGEM vector. The plasmids were linearized with the restriction enzyme *Sal* I, and DIG-cDNA and DIG-hDNA probes, respectively complementary and homologous to CEVd, were synthesized with the appropriate choice of primers. The suitability of these DIG-DNAs to act

as probes was tested by dot-blot hybridization (Fig. 3A). Equimolar concentrations of DIG-cDNA (4.8 nM), DIG-hDNA (4.8 nM), or DIG-cDNA (2.4 nM) plus DIG-hDNA (2.4 nM), produced, all three, the same hybridization patterns when hybridized with non-diluted, ten-fold diluted and hundred-fold diluted plasmid preparations containing the full-length CEVd sequence (Fig. 3A).
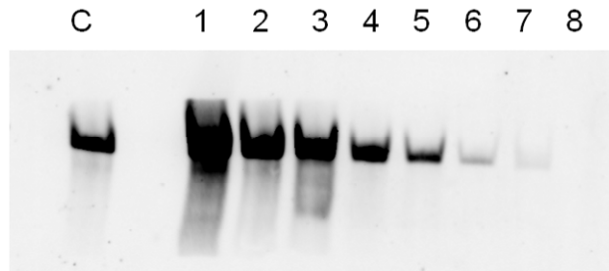
Fig. 2. Analysis of 'Washington navel' sweet orange (**C**) and Etrog citron (1-8) infected with CEVd. Nucleic acid preparations from Etrog citron were included as the following dilutions: (1) 5×10^{-1}, (2) 10^{-1}, (3) 5×10^{-2}, (4) 10^{-2}, (5) 5×10^{-3}, (6) 10^{-3}, (7) 5×10^{-4}, (8) 10^{-4}.

In the plasmid preparation, the full-length CEVd sequences are present as double stranded DNA in which a homologous CEVd sequence (h-CEVd) is annealed to a complementary (c-CEVd) sequence. Thus, DIG-hDNA was able to hybridize with c-CEVd, and DIG-cCEVd with h-CEVd, and the two probes, alone or mixed together, were able to hybridize, and gave the three same hybridization patterns (Fig. 3A). However, in nucleic acid preparations from CEVd infected plant samples, the CEVd sequence is present only as h-CEVd RNA, and when such samples were used for northern hybridizations with the above three equimolar probes, only the DIG-cDNA probe, alone (Fig. 3B, center) or in the presence of DIG-hDNA (Fig. 3B, right), but not the DIG-hDNA probe (Fig. 3B, left), was able to hybridize. This result shows that the presence of DIG-hDNA does not prevent DIG-cDNA from hybridizing, and therefore, in all further assays, the DIG-labelled probe was synthesized by a simple PCR step, even though this synthesis yields both DIG-hDNA and DIG-cDNA.

In an attempt to optimize the hybridization assay, samples separated by PAGE and sPAGE were simultaneously transferred to the same membrane and hybridized with a CEVd-specific probe generated by PCR.

When the hybridization was performed at 60ºC (Fig. 4), the signals from the samples treated by PAGE in a non-denaturing procedure, were always stronger (Fig. 4, lanes 1', 2', 4') than those from the denaturing sPAGE (Fig. 4, lanes 1, 2, 4). This observation was confirmed by estimating by Quantitative Real Time PCR (qPCR), the amount of probe bound in each case.
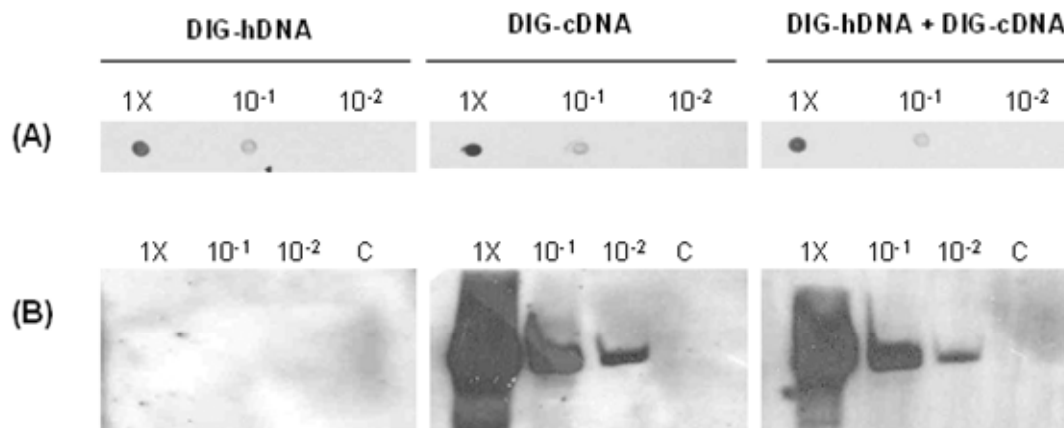
Fig. 3. Hybridization with equimolar amounts of DIG-cDNA, DIG-hDNA and a mixture of DIG-cDNA and DIG-hDNA probes. **(A)** Dot-blot hybridization with 10 fold dilutions of a plasmid containing as an insert the full CEVd sequence. **(B)** Northern hybridization of ten fold dilutions of a nucleic acid preparation from CEVd infected citron. **(C)**=Non-infected control.

Overlapping the membrane with the overexposed film identified the portion of the membrane with the probe bound to the target viroid, and the DNA recovered from each membrane fragment was subjected to qPCR. The amount of DIG-cDNA probe bound to the PAGE-samples was always three to nine times higher (average of 3 estimates: 228,704 copies), than the amount of DIG-cDNA bound to the sPAGE samples (average of 3 estimates: 29,417 copies bound to CEVd-c, the circular form, and 12,972 copies bound to CEVd-l, the linear form). As expected, when the hybridization temperature was increased to 80ºC, the signals obtained were always weak (data not shown) and the amount of probe bound to the PAGE-samples was about half (average of 2 estimates: 46,974 copies) of that bound to the sPAGE samples (average of 2 estimates: 90,211 copies bound to CEVd-c and 8,376 copies bound to CEVd-l). When the hybridization temperature was set at 70ºC, an intermediate situation was found (data not shown).

In summary, even though at 60ºC the number of DIG-cDNA molecules bound to the CEVd in its rod-like secondary structure (PAGE samples) was higher that the number of DIG-cDNA molecules bound to the denatured CEVd-c and CEVd-l (sPAGE samples), at 80ºC larger amounts of probe remained bound to the CEVd-c and CEVd-l than to the rod-like CEVd of the PAGE samples. Also, in the rod-like structure of the PAGE-samples, the binding of the probe was weaker but several copies of the probe were able to bind to a single CEVd molecule. A schematic representation is proposed in Fig. 4.
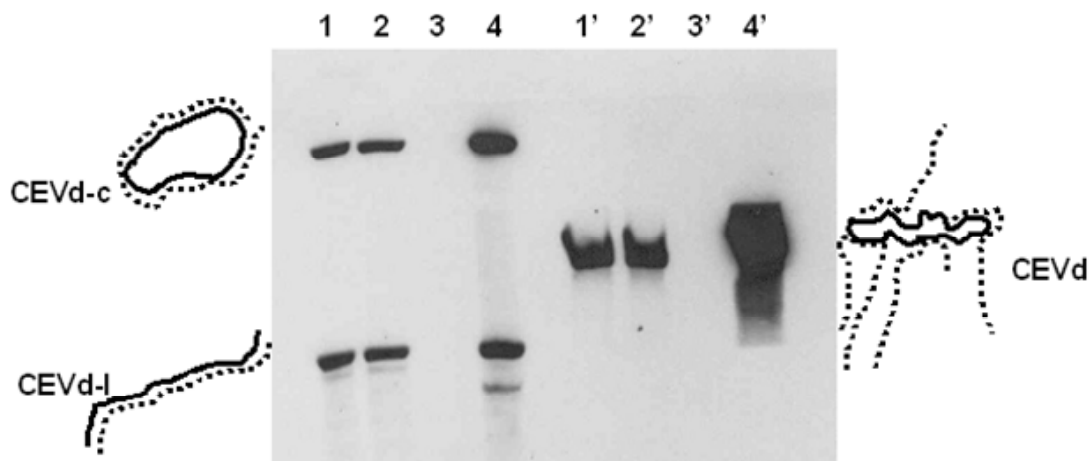
Fig. 4. Northern hybridization analysis of nucleic acid preparations from of CEVd infected plants separated by sPAGE (lanes 1 to 4) and by PAGE (lanes 1' to 4'). Samples separated by PAGE and by sPAGE were transferred simultaneously to the same membrane. Samples are: (1, 2, 1', 2') Infected 'Washington navel' sweet orange; (3, 3') Non-infected 'Washington navel' sweet orange; (4, 4') Infected Etrog citron.

Specific detection of citrus viroids in field grown sweet orange trees, and other citrus species and cultivars

The northern hybridization approach outlined above was tested for the detection of CEVd, CBLVd, HSVd, CDVd and CBCVd in infected, field-grown sweet orange trees in comparison with the classic sPAGE technique. The results from the sPAGE analysis illustrated the low titers of CEVd, CBLVd and CDVd (Fig. 5A: lanes 1, 2 and 4), whereas HSVd and CBCVd were undetectable (Fig. 5A: lanes 3 and 5). Viroids in the two samples from Etrog citron plants, used as positive controls and infected with CEVd and CBCVd (Fig. 5A: lane C) or with CBLVd, HSVd and CDVd (Fig. 5A: lane C'), were all detected.

With the new approach, in which the same samples were subjected to PAGE, transferred to membranes and hybridized with viroid-specific probes, all viroids were readily detected not only in the Etrog citron controls but also in the sweet orange samples (Fig. 5B to F).

No background hybridization was observed, and in all instances the hybridization was highly specific. Moreover, using a mixture of the five probes, viroid infected plants could be discriminated from viroid-free plants in a single hybridization assay (Fig. 5G).

To evaluate the suitability of the method, samples from the viroid infected sweet orange trees were collected at different growing seasons (September 2006; December 2006; March 2007; June 2007). All viroids were detected regardless of the collection time (Fig. 6).

However, the titers of CEVd and CBLVd were low, but still detectable, when the samples were collected in the winter (Fig. 6: lanes 3 and 4).
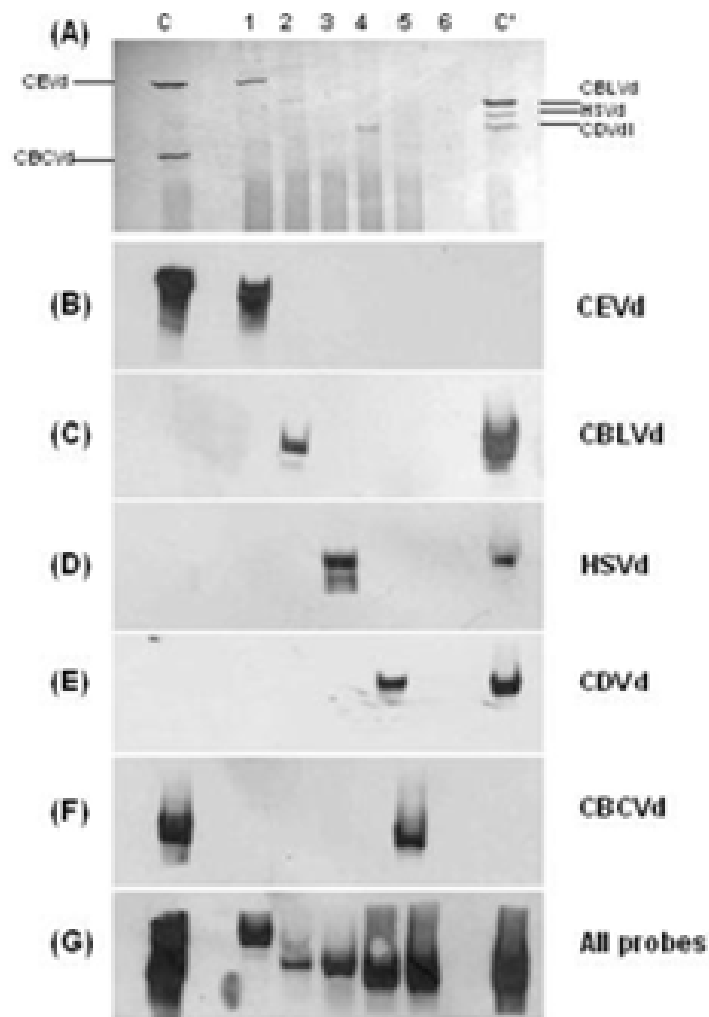
Fig. 5. Analysis of 'Washington navel' sweet orange and Etrog citron infected with citrus viroids by sPAGE **(A)** and northern hybridization with viroid specific probes **(B, C, D, E, F, G).** Sweet orange samples infected with CEVd (1), CBLVd (2), HSVd (3), CDVd (4), CBCVd (5) and non-inoculated control (6). Etrog citron samples co-infected with CEVd and CBCVd (C) and with CBLVd, HSVd and CDVd (C').

The method was further tested to detect viroids from different species and cultivars. Samples of 'Fino' lemon (*C. lemon* (L) Burm.f.), 'Common' mandarin (*C. reticulata* Blanco), 'Tahiti' lime (*C. latifolia* Tan.), 'Foster' grapefruit (*C. paradisi* Macf.) and sour orange (*C. aurantium* L.) known to be infected with CEVd, CBLVd, HSVd, CDVd and CBCVd were analyzed by northern hybridization and the viroids were succesfully detected (Fig. 7). With this method, CEVd has been successfully detected in 'Washington navel' sweet orange (64 samples), 'Valencia' sweet orange (2 samples), 'Rubi' sweet orange (1 sample), Tahiti lime (16 samples) and Mexican lime (*C. aurantifolia* (Christm.) Swing.) (6 samples); CBLVd in 'Washington navel' sweet orange (42 samples), 'Valencia' sweet orange (2 samples), 'Rubi'

sweet orange (1 sample), Tahiti lime (10 samples) and Mexican lime (6 samples); HSVd in 'Washington navel' sweet orange (59 samples), 'Valencia' sweet orange (2 samples), 'Rubi' sweet orange (1 sample), Tahiti lime (10 samples) and Mexican lime (6 samples); CDVd in 'Washington navel' sweet orange (47 samples), 'Valencia' sweet orange (2 samples), 'Rubi' sweet orange (1 sample), Tahiti lime (10 samples), Mexican lime (6 samples) and Swingle citrumelo (*Citrus paradisi* x *Poncirus trifoliata*) (15 samples); and CBCVd in 'Washington navel' sweet orange (29 samples), 'Valencia' sweet orange (2 samples), 'Rubi' sweet orange (1 sample), Tahiti lime (10 samples) and Mexican lime (6 samples). The newly reported CVd-V can also be detected with this method (Serra *et al.*, 2008)
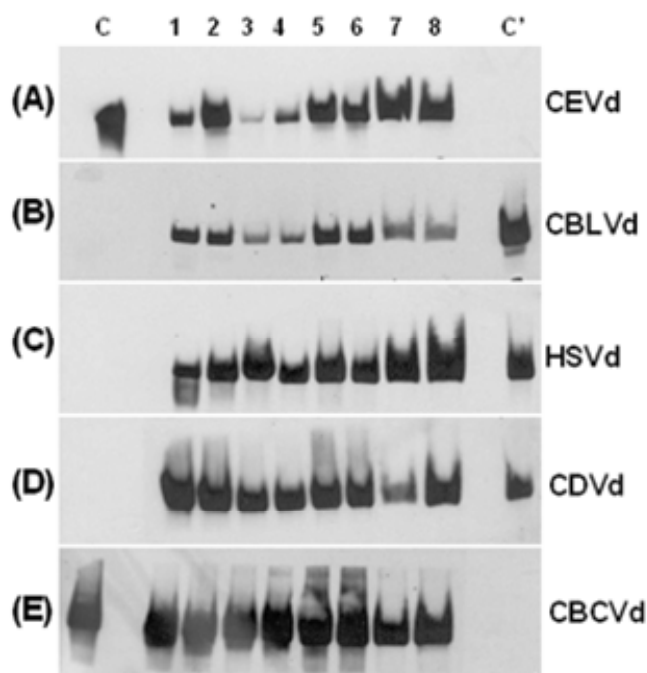
Fig. 6. Northern hybridization analysis of samples of 'Washington navel' sweet orange trees collected in different growing seasons. The samples were collected from the same viroid infected trees in September 2006 (1,2); December 2006 (3,4), March 2007 (5,6); and June 2007(7,8). The sampled trees were infected with CEVd (**A**), CBLVd (**B**), HSVd (**C**), CDVd (**D**) or CBCVd (**E**). Samples of greenhouse grown Etrog citron plants co-infected with CEVd and CBCVd (**C**) and with CBLVd, HSVd and CDVd (**C'**) were included as positive controls.

Hybridisation protocol for routine analysis

The hybridization protocol involves the following steps: (i) collection of samples for nucleic acid extraction (bark yields similar or higher viroid titers than leaves); (ii) tissue homogenization in phenol containing extraction buffer followed by 2M LiCl partition to yield viroid-enriched preparations; (iii) separation in 5% PAGE followed by ethidium bromide staining (a lane containing the 100 bp molecular weight marker is desirable to identify the viroid- containing region in the gel); (iv) excision of the fragment of the gel (containing the

200 and 400 bp markers), electroblotting to membrane and fixing; (v) hybridization with viroid specific DIG-labelled probe (or a mixture of several probes) at 60ºC; (vi) detection with anti-DIG-alkaline phosphatase conjugate and autoradiography with the CSPD substrate.
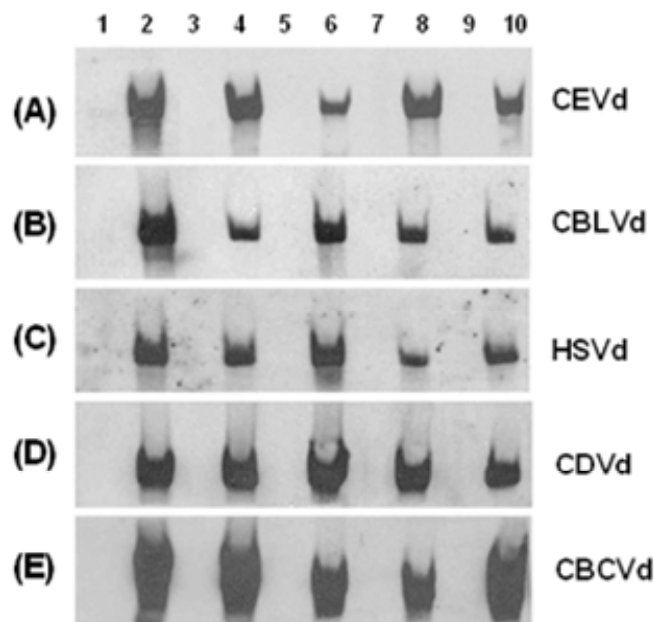
Fig.7. Northern hybridization with viroid-specific probes of different species of citrus co-infected with CEVd, CBLVd, HSVd, CDVd and CBCVd. Infected plants of 'Fino' lemon (2), 'Common' mandarin (4), 'Tahiti' lime (6), 'Foster' grapefruit (8) and sour orange (10) and the corresponding healthy controls (1, 3, 5, 7, 9).

Several of these steps can by simplified as follows:

Collection and storage of samples. Fresh samples can be processed immediately after collection or stored at –20ºC for several months or even several years. Viroids can be successfully detected from preparations obtained using dry samples that lost water during storage at room temperature or during transport (wet paper inside the collection bags should be avoided to prevent the tissue to rot). The northern hybridization results obtained with dry samples that have been kept in the bench for more than a week are shown in Fig. 8.

Electrophoresis and blotting. Nucleic acids can be electrophoresed in 1% agarose gels (in TBE buffer for 60 min, at 100 V instead of 5% PAGE). As in PAGE, the RNAs in the fragment of the gel (containing the 200 and 400 bp markers) can transferred to a membrane by electroblotting or by capilarity diffusion. The northern hybridization results obtained with samples separated in 1% agarose are shown in Figure 9, and are comparable to those shown in Fig. 5.
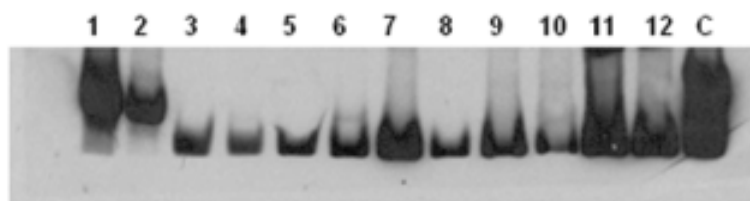
Fig. 8. Northern hybridization analysis of samples of 'Washington navel' sweet orange with a mixture of CEVd, CBLVd, HSVd, CDVd and CBCVd specific probes. Tissue samples had been collected from field grown trees infected with CEVd (1,2), CBLVd (3,4), HSVd (non-cachexia inducing variant CVd-IIa) (5,6), HSVd (cachexia inducing variant CVd-IIc) (7,8), CDVd (9,10) or CBCVd (11,12) and stored at room temperature for two weeks. Sample of greenhouse grown 'Fino' lemon co-infected with CEVd, CBLVd, HSVd, CDVd and CBCVd (C) was included as positive control.
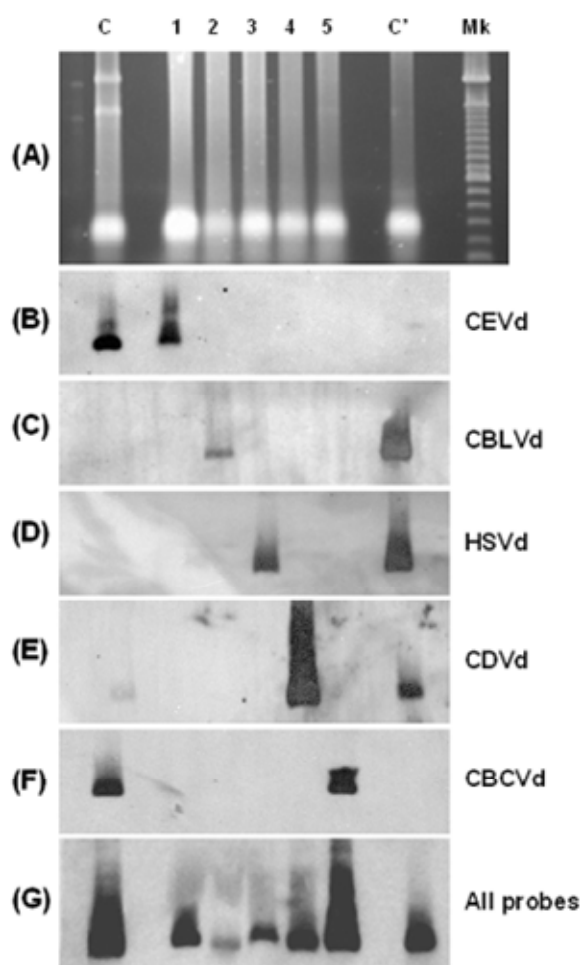
Fig. 9. Analysis of 'Washington navel' sweet orange and Etrog citron infected with citrus viroids by electrophoresis in 1% agarose **(A)** and northern hybridization with viroid specific probes (**B, C, D, E, F, G)**. Sweet orange samples infected with CEVd (1), CBLVd (2), HSVd (3), CDVd (4), CBCVd (5) and non-inoculated control (6). Etrog citron samples co-infected with CEVd and CBCVd **(C)** and with CBLVd, HSVd and CDVd **(C')**. Mk = 100bp molecular weight marker.

Conclusion

Viroid control is critical for the commercial propagation of budwood released from quarantine, sanitation and certification programs. These programs require large numbers of indexing tests, and these tests must be as sensitive and as economic as possible. The use of the viroid amplification host, Etrog citron, coupled to sPAGE or hybridization analysis, was proposed previously (Palacio *et al.*, 2000; Duran-Vila; *et al.*, 1993) and has been used as the most sensitive alternative to the conventional methods based solely on biological indexing. The main drawback of this approach is the high cost associated with inoculation and maintenance of Etrog citron plants for 3 to 6 months at the high temperatures (28-32ºC) required for viroids to reach detectable titers.

Up to now, viroid detection directly in commercial citrus species and cultivars has been achieved in a reliable way only with PCR-based approaches. Unfortunately, false positives due to amplicon contamination and false negatives due to the failure to generate a cDNA of suitable size during reverse transcription, are not infrequent, and preclude the use of RT-PCR for large scale indexing. The northern hybridization approach described here does not require Etrog citron as an amplification host and provides an alternative for reliable viroid indexing. The hybridization procedure and synthesis of probes is easy to handle and can be successfully used for viroid detection in samples collected from commercial species and cultivars collected in different growing seasons. Like in the case of RT-PCR, the sensitivity of northern hybridization is associated with a high specificity and can only be used to detect known viroids for which probes are available. Therefore in the case of samples introduced from abroad that may carry unknown viroids, the northern hybridization technique has to be complemented by sPAGE and silver staining in which detection is not based on a specific reagent.

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Capítulo II

Citrus viroids in Colombia

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Abstract

A sensitive northern hybridization method developed to detect viroids in commercial species and cultivars was used to start surveying for citrus viroids in orchards from Colombia. Analysis of seven samples collected in the Tolima Region showed that five Tahiti lime sources were infected with HSVd and CDVd, whereas two Mexican lime sources were viroid-free. Three additional Mexican lime sources collected in Magdalena municipality were also viroid-free, whereas two Tahiti lime sources were infected with HSVd and CDVd or CEVd, HSVd and CDVd. Cultivars of Valencia sweet orange, Mexican lime and Tahiti lime, maintained in the germoplasm collection of Palmira, were all viroid-free. Of the three citron sources, also maintained in the germoplasm collection, two were viroid-free and one was infected with CEVd and CDVd. The sequences of the viroids from the infected sources were determined and compared with the reference sequences. Two nucleotide changes (A315$\rightarrow$G and U316$\rightarrow$A) were identified in a CEVd variant recovered from a symptomless Etrog citron. Experiments in course using two synthetic mutants affecting these nucleotide positions will reveal their implication in suppressing symptom expression.

Introduction

Colombia is a citrus growing country with an estimated production of 1.1 million tons on a surface of 57,420 has. Tahiti lime is the main export commodity, accounting for 12.6% of the overall citrus growing area (MADR, 2005) with a productivity of only about 8 metric tons/ha in small orchards, much below the 15-25 metric tons/ha that can be reached in well managed commercial plantations of Colombia and other countries of the region (Toro *et al.*, 2002). *Citrus tristeza virus* (CTV), including stem-pitting isolates, is endemic in Colombia (Murcia *et al*., 2002; Murcia *et al*, 2005) where it causes important economic losses. The presence of other graft-transmissible diseases has been reported on the basis of symptoms on sensitive rootstocks and cultivars. The need for high quality planting material was acknowledged by ICA (Instituto Colombiano Agropecuario) and numerous international experts recommended the implementation of a "Plan Nacional de Certificación de Material de Propagación de Cítricos" as a way to maintain and improve competitiveness of the citrus sector.

Grafted citrus trees may be infected with viroids without showing any signs of infection when both scions and rootstocks are tolerant to the viroids involved. Tahiti and Mexican limes on Volkamer lemon, widely grown in Colombia, are such tolerant scion/rootstock combinations. Here we report the results of a preliminary survey for citrus viroid identification in three municipalities of Colombia.

Materials and methods

Plant materials and nucleic acid extraction

Samples of Tahiti lime (10 samples), Mexican lime (6 samples), Valencia sweet orange (1 sample) and citron (3 samples) were collected in Tolima, Magdalena and Palmira municipalities (Table 1). Bark (5 g) stripped from young shoots was reduced to powder in liquid nitrogen and homogenized in 5 ml of extraction buffer (0.4M Tris-HCl pH 8.9; 1% (w/v) SDS; 5mM EDTA pH 7.0; 4% (v/v) 2-mercaptoethanol) and 15 ml of water-saturated phenol (Semancik, *et al*., 1975). The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1mM MgCl$_2$ pH 7.4). Aliquots of these preparations were used for northern blot hybridization and RT-PCR analysis.

Northern blot hybridization

Aliquots (20 µl equivalent to 300 mg of fresh weight tissue) of the nucleic acid preparations were subjected to 5% PAGE (39:1) under non-denaturing conditions (2 h, 60 mA) (Morris and Wright, 1975). The RNAs were then electroblotted (400 mA for 2 h) from the gel to positively-charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) and immobilized by UV cross-linking. DIG-labeled viroid-specific probes were synthesized by PCR using cloned plasmids containing full-length viroid DNAs as described by Palacio-Bielsa *et al.* (2000). Prehybridization (at 60ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and 6×SSC buffer (SSC: 150 mM NaCl; 15 mM sodium citrate) containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking solution (Roche). After hybridization the membranes were washed twice in 2×SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1×SSC, 0.1% SDS at 60ºC for 60 min. The DIG-labelled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualized with the chemiluminiscence substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate (CSPD) (Roche), and revealed by autoradiography.

RT-PCR analysis

Retrotranscription and PCR amplification was performed as described by Bernad and Duran-Vila (2006). First-strand cDNA was synthesized at 60ºC using 27-mer primers specific for each viroid and Thermoscript reverse transcriptase (Invitrogen®). Full-length viroid DNAs were recovered performing second strand synthesis and DNA amplification with sets of contiguous 18-mer forward and reverse primers specific for each viroid in 50 µl reactions

containing the PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH8.3), 1.5 mM MgCl$_2$, 0.12 mM dNTPs, 0.5 μM of each primer and 1 U of *Taq* DNA polymerase. Electrophoretic analysis in 2% agarose gels confirmed the synthesis of the expected DNA products.

Sequencing and sequence analysis

Whenever possible, full-length uncloned viroid amplicons were sequenced. When the sequencing results were unclear, the amplicons were purified (GFX^{TM} PCR-DNA and Gel Band Purification Kit, Amersham Bioscience) and ligated to the pGEM-T vector (Promega). *Escherichia coli* cells (strain DH5α) were used for plasmid cloning. Uncloned amplicons and plasmid inserts were sequenced with the ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple sequence alignments were performed with Clustal W (Thompson *et al.*, 1994). Nucleotide distances were estimated considering alignment gaps and using the Jukes and Cantor's method (Jukes and Cantor, 1969) for correction of superimposed substitutions with the MEGA 3.1 program (Kumar *et al.*, 2004). Phylogenetic tree was obtained with Neighbor-joining method (Saitou and Nei, 1987) using the MEGA 3.1 program (Kumar *et al.*, 2004).

Site directed mutagenesis

A PCR-based protocol (Byrappa *et al.,* 1995) was followed with minor modifications (Gago *et al.*, 2005). Briefly, plasmid pCEVd^{CO} (containing the full length sequence of the predominant variant of the CEVd isolate form Colombia) and pCEVd-117-V1 (containing the full length sequence of variant V1, GenBank AJ54795) (Gandía *et al.,* 2005) (5 ng each) were amplified with 250 ng each of pairs of adjacent phophorilated primers. Appropriated changes were introduced in the forward primers F-V1 (5'**AT**ATCTTCACTGCTCTCCGGGCG 3') and F-CEVd^{CO} (5'**GA**ATCTTCACTGCTCTCCGGGCG 3') to obtain the desired mutants. The same reverse primer CEVd-MUT-R (5'AAGAAAAGCGGTTTGGGGGTTGAAGC 3') were used in both cases. The PCR cycling profile designed to amplify the complete plasmid with Pfu Turbo DNA polymerase consisted of 30 cycles of 30 s at 94°C, 30 s at 60°C and 3.5 min at 72°C, with an initial denaturation at 94°C for 2 min and a final extension at 72°C for 10 min. After electrophoresis in 1% agarose gels, PCR-amplified products of plasmid length, purified with the QIAquick kit (QIAGEN), were circularized with T4 ligase, and used for transformation. Sequencing confirmed that the plasmids contained only the desired mutations.

Infectivity and bioassay of mutants.

Monomeric mutated and non-mutated CEVd-DNA inserts were recovered as blunt-end PCR products using phosphorylated primers CEVd-R1 and CEVd-F1 (Bernad *et al.*, 2006) and Pfu DNA polymerase. The DNA products were subjected to ligation with 2 U of T4 DNA

ligase (Gibco) and the dimeric molecules were cloned in pBluescript II KS (+) digested with EcoRV. Sequencing confirmed that the plasmids contained the desired head-to-tail orientation of the dimeric inserts. Clones with these inserts were linearized with *Hind*III and used as a template in a transcription reaction with 1 mM NTPs, 1 mM DTT and 50 U of T7 RNA polymerase to produce dimeric transcripts homologous to the viroid sequence. Three Etrog citron seedlings were slash-inoculated (50 ng of transcript per plant) and kept in the greenhouse at 28º-32ºC. Infection was assessed by sPAGE and northern blot hybridization analysis.

Results

Eight of the twenty samples analyzed showed a positive hybridization with some of the probes (Table 1). All the Mexican lime sources were viroid-free whereas seven out of ten Tahiti lime sources were infected with HSVd and CDVd or with CEVd, HSVd and CDVd. One out of three citrons tested was infected with CEVd and CDVd. Valencia sweet orange was viroid-free. Viroid infection was confirmed by RT-PCR. The consensus sequence of these viroid isolates was determined by sequence analysis of the uncloned RT-PCR amplicons or the inserts of recombinant plasmids.

Characterization of CEVd isolates

The uncloned amplicons from the two CEVd sources gave ambiguous results and the consensus sequences were obtained from four clones of each isolate. The consensus sequences recovered from Tahiti lime and from Etrog citron presented the highest identity (94.9% and 96.5%) with the reference sequences of class A defined by Visvader and Symons, (1985; 1986). Sequence alignment showed that CEVd from Tahiti lime differed in 14 nucleotide changes from the reference sequence of Class A but maintained the characteristic sequence of the PL motif located in the Pathogenicity (P) domain.

However, a number of changes (four in the upper strand and three in the lower strand of the CEVd secondary structure) were identified in the region of the P_R motif located in the Variable (V) domain. Three of the remaining changes located in the lower strand of the Central (C) and P domains had also been identified in a CEVd isolate previously described by (Gandía *et al*., 2005). CEVd from citron differed in 8 nucleotide changes from the reference sequence of Class A that included A315→G and U316→A changes in the lower strand of P_L motif located in the P domain. None of the changes affected the P_R motif located in the V domain. From the remaining changes four had been identified in a CEVd isolate previously described (Gandía *et al*., 2005). As illustrated in the phylogenetic tree obtained with the Neighbor-Joining method (Fig. 1), the two CEVd sequences clustered with CEVd-A.

Table 1. Viroids in Citrus samples collected in Colombia Municipalities

Municipality	Scion	Rootstock	CEVd	HSVd	CBLVd	CDVd	CVd-IV	CVd-V
Tolima	Tahiti lime 1	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 2	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 3	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 4	Volkamer lemon	-	+	-	+	-	-
Tolima	Tahiti lime 5	Volkamer lemon	-	+	-	+	-	-
Tolima	Mexican lime 1	Volkamer lemon	-	-	-	-	-	-
Tolima	Mexican lime 2	Volkamer lemon	-	-	-	-	-	-
Magdalena	Tahiti lime 6	Carrizo citrange	+	+	-	+	-	-
Magdalena	Tahiti lime 7	Volkamer lemon	-	+	-	+	-	-
Magdalena	Tahiti lime 8	Volkamer lemon	-	-	-	-	-	-
Magdalena	Tahiti lime 9	Cleopatra mandarin	-	-	-	-	-	-
Magdalena	Mexican lime 3	Volkamer lemon	-	-	-	-	-	-
Magdalena	Mexican lime 4	Volkamer lemon	-	-	-	-	-	-
Magdalena	Mexican lime 5	Volkamer lemon	-	-	-	-	-	-
Palmira	Tahiti lime 10	Unknown	-	-	-	-	-	-
Palmira	Mexican lime 6	Unknown	-	-	-	-	-	-
Palmira	Valencia sweet or.	Unknown	-	-	-	-	-	-
Palmira	Etrog citron 1	Unknown	+	-	-	+	-	-
Palmira	Etrog citron 2	Unknown	-	-	-	-	-	-
Palmira	Citron	Unknown	-	-	-	-	-	-
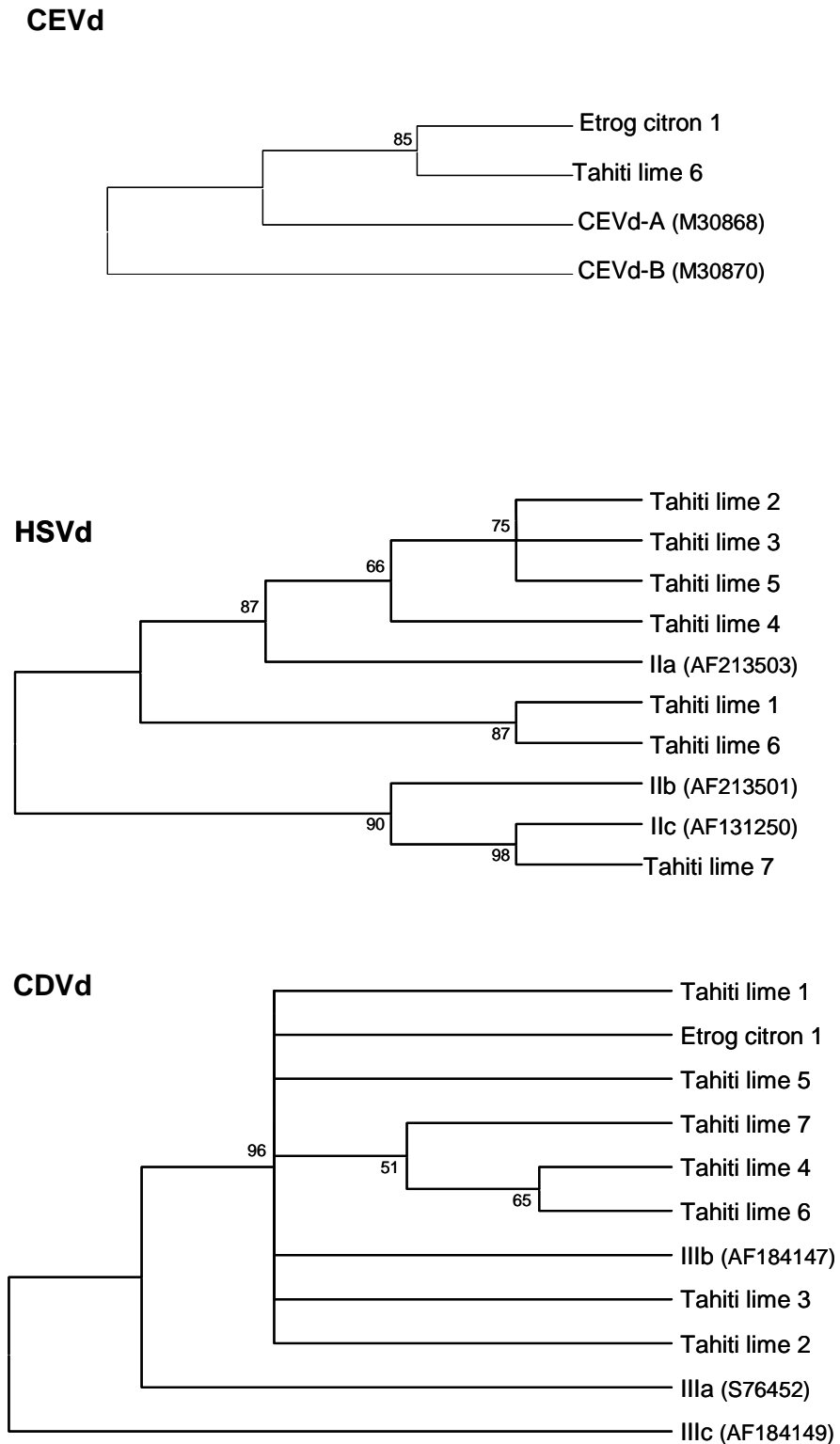
CEVd

HSVd

CDVd

Fig. 1. Neighbor-joining phylogenetic tree obtained with the CEVd, HSVd and CDVd sequences recovered from Tahiti lime and Etrog citron with 10000 replicates. Nucleotide distances were estimated using the Jukes and Cantor's method (Jukes and cantor ,1969). Condensed branches have bootstrap values lower than 50%. Reference sequences were included in each case: CEVd the reference sequences of Class A and Class B (Visvader and Symons, 1985; 1986); HSVd reference sequences of CVd-IIa, CVd-IIb and CVd-IIc (Reanwarakorn and Semancik, 1998); CDVd reference sequences CDVd-IIIa, CDVd-IIIb and CDVd-IIIc (Rakowski, *et al.*, 1994; Semancik *et al.*, 1997).

Characterization of HSVd isolates

The consensus sequences of the seven HSVd isolates were obtained by sequencing the uncloned RT-PCR amplicons. The five HSVd isolates recovered from Tahiti lime trees (Tahiti lime 1 to 5) collected in Tolima municipality presented the highest sequence identities (99.3-99.6%) with the non-cachexia variants (CVd-IIa) of HSVd (Table 2) differing from the reference sequence in 1-2 changes. They all presented, within the Variable (V) domain, the six nucleotide motif characteristic of non-cachexia sequence variants of this viroid (Palacio-Bielsa *et al*., 2004; Reanwarakorn and Semancik, 1998). One of the two HSVd isolates recovered from Tahiti lime (Tahiti lime 6) collected in Magdalena municipality, also presented the highest sequence identity (98.0%) with the non-cachexia variants (CVd-IIa) (Table 2). This sequence variant however, presented only four of the six nucleotides discriminating non-cachexia from cachexia inducing sequence variants. It presented two compensatory deletions, $-A(116)$ and $-U(189)$, in the upper and lower strands of the V domain, which being characteristic of cachexia variants, were reported previously in a non-cachexia isolate from Cuba (Velázquez *et al*., 2002) . The HSVd isolate recovered from Tahiti lime (Tahiti lime 7) also collected in Magdalena municipality presented the six-nucleotide motif characteristic of cachexia sequence variants with the highest sequence identity (99.6%) with CVd-IIc variants (Reanwarakorn and Semancik, 1998). As illustrated in the phylogenetic tree obtained with the Neighbor-Joining method (Fig 1), all except one of the HSVd isolates clustered with the non-cachexia variant (CVd-IIa).

Characterization of CDVd isolates.

The consensus sequences of four CDVd isolates (Tahiti lime 3, 5, 6, 7) were obtained by sequencing the uncloned RT-PCR amplicons whereas the remaining (Tahiti lime 1, 2, 4 and Etrog citron 1) were obtained from the sequences of three to four clones from each isolate. All the sequences presented the highest sequence identities (ranging from 99.0 to 100%) with CVd-IIIb (Rakowski *et al*., 1994) (Table 2). The consensus sequences of three isolates (Tahiti lime 2, 3 and Etrog citron 1) were identical to the reference sequence of CVd-IIIb defined by Rakowski *et al*., (1994). The reference sequences of the other five isolates differed from 2 to 7 changes from the reference sequence of CVd-IIIb (Rakowski *et al*., 1994) and the changes were found to be located in different regions of the secondary structure of the viroid molecule: V domain (Tahiti lime 1), T_R domain (Tahiti lime 4), P domain (Tahiti lime 5) and T_L (Tahiti lime 6, 7). As illustrated in the phylogenetic tree obtained with the Neighbor-Joining method (Fig. 1), all the CDVd isolates clustered with the variant CVd -IIIb described by Rakowski *et al*.,(1994).

Table 2. Sequence identities of viroids identified in Colombia with the type members of CEVd, HSVd and CDVd.

Sample	Sequence identities (%)							
	CEVd		HSVd			CDVd		
	CEVd-A	CEVd-B	CVd-IIa	CVd-IIb	CVd-IIc	CDVd -IIIa	CDVd -IIIb	CDVd -IIIc
	(M 30868)	(M 30870)	(AF213503)	(AF213501)	(AF131250)	(S76452)	(AF184147)	(AF184149)
Tahiti lime 1	-	-	**99.6**	97	93.7	95.3	**99.3**	95
Tahiti lime 2	-	-	**99.3**	97	93.7	96	**100**	95.2
Tahiti lime 3	-	-	**99.3**	97	93.7	96	**100**	95.6
Tahiti lime 4	-	-	**99.6**	97.3	93.7	95.3	**99.3**	94.5
Tahiti lime 5	-	-	**99.3**	97	93.7	95.3	**99.3**	95
Tahiti lime 6	**94.9**	92.5	**98**	96.7	93.7	94.3	**99**	93.8
Tahiti lime 7	-	-	93.7	95.7	**99.6**	95	**99.3**	94.2
Etrog citron 1	**96.5**	93	-	-	-	95.6	**100**	95.2

Mutagenesis approach to identify nucleotide positions involved in CEVd patogenesis.

The CEVd identified in an apparently symptomless Etrog citron tree from the Germplasm Bank of Palmira municipality contained two mutations in the P_L motif. Since the P_L motif is responsible for the modulation of symptom expression (Visvader and Symons, 1985; 1986), further characterization of this peculiar CEVd strain should reveal interesting information on the pathogenicity of CEVd. Mutagenesis experiments were conducted to elucidate if the two changes identified in the P_L motif of the CEVd^{CO} isolate were responsible for the lack of symptoms in infected Etrog citron. Two artificial CEVd mutants were synthesized: (i) mutant MCEVd^{CO} was produced by incorporating two substitutions (G313$\rightarrow$A and A314$\rightarrow$U) to shift the composition of the P_L motif of CEVd^{CO} to that characteristic of Class A variants; (ii) mutant MCEVd-117-V1 was produced by incorporating two substitutions (A313$\rightarrow$G and U314$\rightarrow$A) to shift the composition of the P_L motif of CEVd-117-V1 which is characteristic of Class A variants to that of CEVd^{CO}. Three Etrog citron seedlings were slash-inoculated with 50 ng of dimeric head-to-tail mutant transcripts per plant. Dimeric head-to-tail transcripts of the natural CEVd-117-V1 and CEVd^{CO} variants, were also inoculated to three Etrog citron plants each, as positive controls. All these plants were kept in the greenhouse at 28–32 ∘C over a 6-month period together with three additional non-inoculated Etrog citron plants as negative controls. Infection was confirmed by northern blot hybridization (data not shown) and the stability of the progeny was assessed by sequencing RT-PCR amplicons from each plant. In order to monitor viroid induced symptoms all the plants were cut at the level of the second citron internode and the second flush of tissue is in the process of evaluation for growth and symptom expression.

Discussion

The results of the survey show that CEVd, HSVd and CDVd are present in Colombia, affecting at least some commercial groves of Tahiti lime. All the HSVd sources have been characterized as non-cachexia strains. The CDVd sources, which seem to be widespread in Tahiti lime groves, present high identities with CDVd-IIIb (Rakowski *et al*., 1994) but contain changes affecting the regions that correspond to four structural domains of the viroid secondary structure.

Even though HSVd and CDVd are the most widespread viroids in Tahiti lime, CEVd is also present at least in one of the sources. Since bark-cracking symptoms in Tahiti lime are not infrequent in Colombia, viroid infections could be the cause (Murcia *et al*., 2009). In addition, CEVd infection of trees also co-infected with HSVd and CDVd may produce undesirable effects, such as those reported by Vernière *et al*., (2004, 2006) in the case of trees

grafted on the viroid-sensitive trifoliate orange. One of the viroid infected Tahiti lime sources from Magdalena municipality (Tahiti lime 6) was obtained from a tree grafted on Carrizo citrange, which is also viroid-sensitive, and therefore such trees may suffer from the sensitivity of both scion and rootstock.

Unexpectedly, the analysis of two symptomless Etrog citron plants maintained in Palmira revealed that one was infected with CEVd and CDVd. Sequence analysis showed that CEVd presented a high sequence similarity with the reference sequence of class A known to induce severe symptoms no only in tomato (Visvader and Symons, 1985; 1986) but also in Etrog citron (Gandía *et al.,* 2005). Interestingly, two of the changes identified (A315→G and U316→A) affected the P_L motif located in the P domain which is a pathogenicity determinant. Experiments in course will reveal if these two nucleotide changes are sufficient to shift a severe variant into a latent one.

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Capítulo III

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 present bark-cracking symptoms, which 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 seven clones that displayed characteristic bark-cracking symptoms were found to be infected with CEVd (a single clone from Brazil), with CEVd and CDVd (two clones from Brazil) or with CEVd, HSVd and CDVd (four clones from Peru), whereas the clones that did not present bark-cracking symptoms were either viroid-free or infected only with CDVd. An assay is being conducted to establish if viroids, and in particular CEVd, are indeed the cause of "quebra-galho" bark cracking symptoms. Preliminary observations showed that two-year old CEVd infected lime trees at Moncada, Spain, presented cracks.

Introduction

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 present desirable traits such as small size suitable for high density planting, easy management (harvesting, phytosanitary treatments, etc), multiple flowering, and production periods at which citrus 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, which limit the use of 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 on this rootstock the bark scaling symptoms characteristic of exocortis disease. However the cause-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 an assay 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 in a farm near Araraquara in the state of São Paulo, Brazil, and displaying the characteristic bark cracking symptoms (Table1, samples 1, 2, and 3). Eight additional samples were also 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 collected 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 nine months later.

Table 1. Identification of citrus viroids in Tahiti lime

Source	Bark cracking	Citrus viroids[a]					
		CEVd	HSVd	CBLVd	CDVd	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.

Viroid Analysis

Citron samples of young stems and leaves (5 g) were powdered in liquid nitrogen and homogenized in 5 ml of extraction buffer (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 (Semancik *et al.*, 1975). 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, aliquots (20 μl equivalent to 300 μg fresh weight) were first subjected to a 5% non denaturing polyacrilamide gel electrophoresis (PAGE) at 60mA for 2.5 hours. 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 hours (Rivera-Bustamante *et al.,* 1986) Viroid bands were visualized by silver staining (Igloi ,1983).

For Northern blot hybridization, aliquots (20 µl 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.*, (2000) for *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark craking viroid* (CBCVd). A probe specific for the newly described *Citrus viroid V* (CVd-V) (Serra *et al.*, 2008) was synthesized using primers CVd-V-h (5'-TCGACGAAGGCCGGTGAGCA-3') and CVd-V-c (5'-CGACGACAGGTGAGTACTCTCTAC-3') respectively 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 2X SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1X SSC, 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, (2006). 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 using a set of two contiguous 18-mer forward and reverse primers specific for each viroid in 50 µl reaction volume containing the PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH8.3), 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 (Thompson *et al.,* 1994).

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 6 viroid sources that had been maintained in Etrog citron (four plants per viroid treatment and four non-inoculated controls). The isolates chosen were CEVd (CEVd-117) (Gandía *et al.*, 2005), CBLVd (CVd-Ia-117) (Foissac and Duran-Vila, 2000), HSVd (X-707 and CVd-IIa-117) (Palacio-Bielsa *et al.*, 2004), CDVd (Foissac and Duran-Vila, 2000), and CBCVd (CVd-IV-Ca) (Francis *et al.*, 1995). CEVd-117 had been characterized as a severe strain (Gandía *et al.*, 2005) highly homologous to the CEVd sequences defined by Visvader and Symons (Visvader and Symons, 1985;1986) as class A. HSVd isolates (X-707 and CVd-IIa-117) had been characterized as cachexia and non-cachexia inducing variants respectively (Palacio-Bielsa *et al.*, 2004).

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 was confirmed by sPAGE analysis in October 2007.

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 presented 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) associated with the "wood pocket syndrome" (Knorr and Childs, 1957).

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 eleven Tahiti lime sources revealed the presence of CEVd, HSVd and CDVd (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) containing in

addition CDVd. 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 CDVd. Three of the symptomless clones collected in Peru (samples 4, 6, and 10) were free of viroids, while only one (sample 9) contained CDVd.
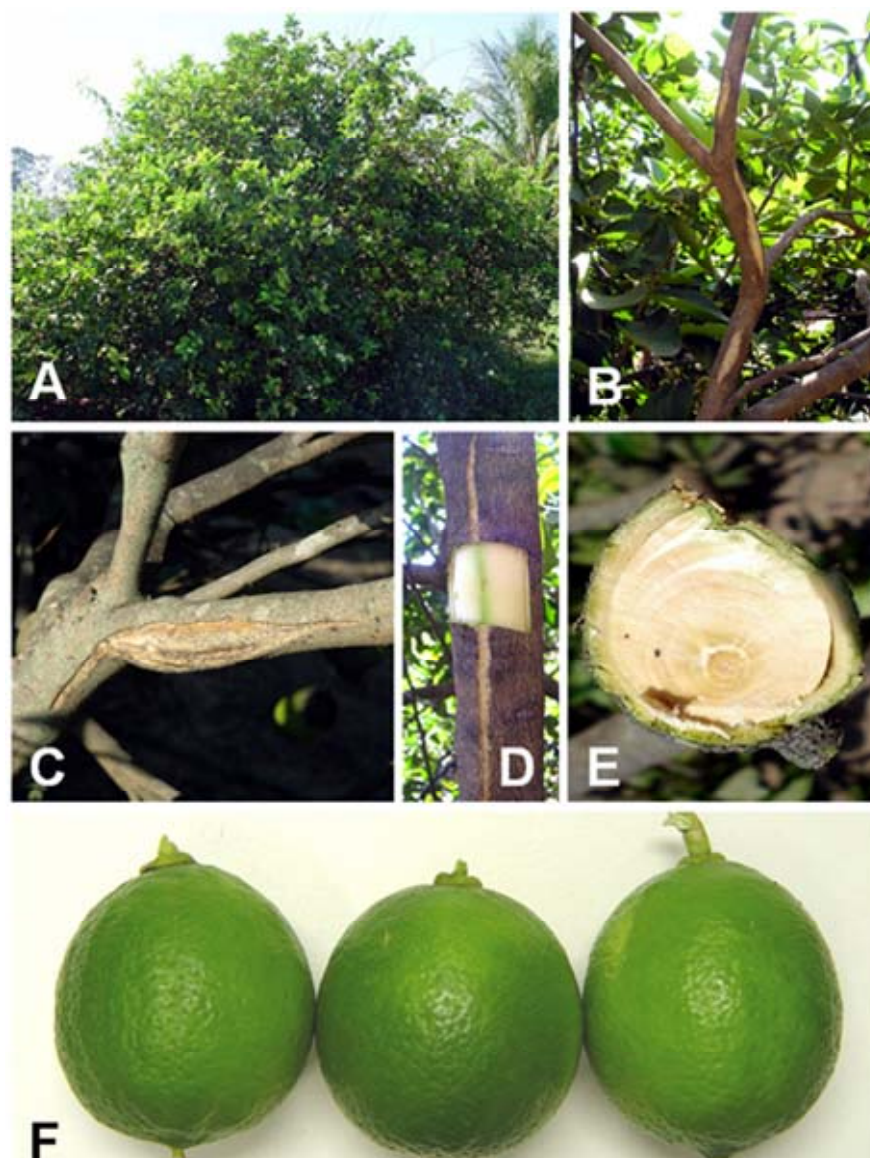
Fig 1. Symptoms in Tahiti lime trees sampled in Brazil and Peru: **(A)** Non-declining tree from Brazil; **(B-C)** Bark cracking symptoms in trees sampled in Brazil and Peru; **(D-E)** Lack of wood staining in trees sampled in Brazil and Peru; **(F)** lack of fruit sectoring symptoms.
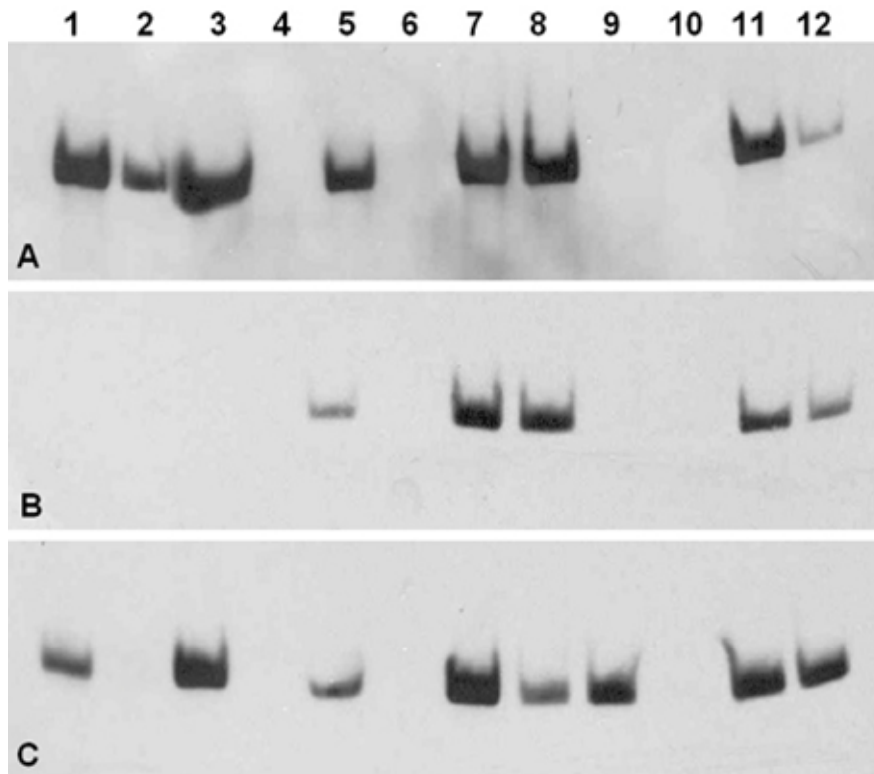
Fig. 2. Positive Northern blot hybridization analyses of Tahiti lime trees using specific probes for CEVd **(A)**, HSVd **(B)** and CDVd **(C).** Samples from Brazil: 1 to 3; samples collected in Peru: 4 to 11, including two samples of Mexican origin (samples 4 and 6); positive viroid control: 12.

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, (1985, 1986). 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 (1985, 1986). 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 CDVd specific primers showed that the two CDVd infected samples from Brazil (samples 1 and 3) contained closely related CDVd sequences, with nucleotide identities ranging from 98.0 to 98.8% with the reference sequence of CDVd-IIIa, as defined by Rakowski *et al.*, (1994). The five CDVd infected samples from Peru (samples 5, 7, 8, 9, and 11) contained very closely related CDVd sequences, with nucleotide identities ranging from 99.3 to 100% with the reference sequence of CDVd-IIIb, as also defined by Rakowski *et al.*, (1994). These results confirmed that the

CDVd infected Tahiti lime clones from Brazil and Peru had different origins. 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 a nucleotide identity of 99.3% with the reference sequence of CVd-IIa, as defined by Reanwarakorn and Semancik, (1998) 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 either CEVd, HSVd, CBLVd, CDVd or CBCVd. The grafted trees were established in an experimental plot at Moncada. After two years, the Tahiti lime scions of the trees presented 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 CBCVd), 9.0 (cachexia HSVd), 10.5 (CDVd) and 76.3 (CEVd). One way ANOVA analysis showed that only the data collected on CEVd infected trees were significantly different from those of all the other treatments and the non-inoculated control (P-value=0.0001<0.05).

Discussion

Assays to determine the cause of bark cracking of Tahiti lime were initiated in 1961 by Salibe and Moreira, (1965) 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 (Salibe and Moreira, 1965). This early work, carried out at a time when viroids had not yet been identified, clearly demonstrated that: (i) a graft-transmissible agent, supposed to be 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 (1943) 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, an affection similar to wood pocket of lemon (Calavan, 1957; Knorr *et al*., 1957), which is presently considered to be a physiological or genetic disorder of certain Tahiti lime clones.
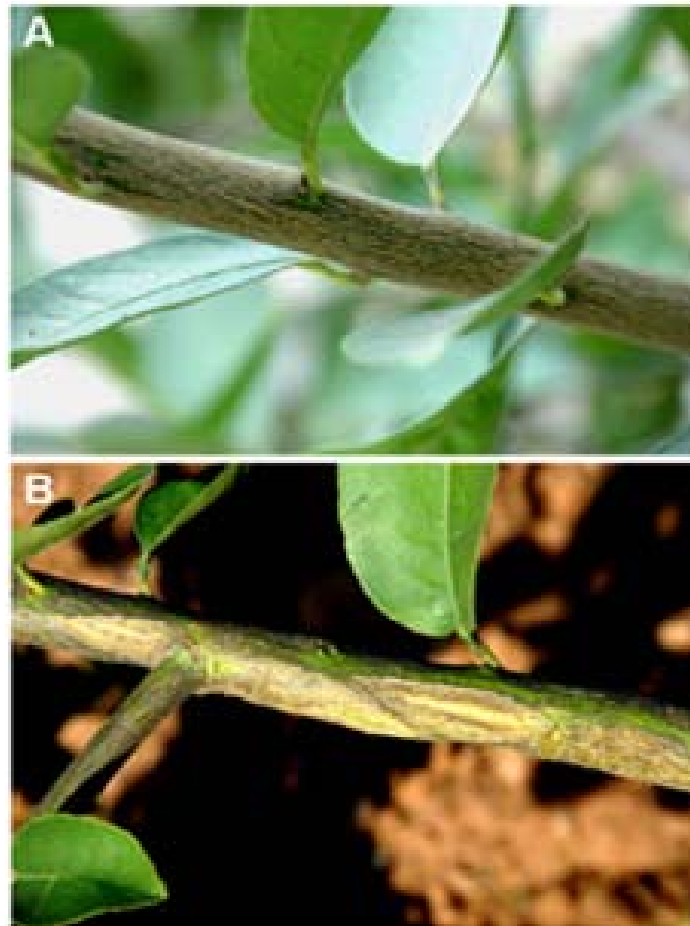
Fig. 3. (A) Viroid-free, symptomless, mature Tahiti lime tree devoid of bark cracks used for graft propagation of lime buds on CEVd infected Cleopatra mandarin rootstock seedlings. **(B)** Bark cracking symptoms observed on the CEVd infected Tahiti lime trees on Cleopatra mandarin growing in the field since 2005.

The work of Salibe and Moreira, (1965) 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: (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 (Alvarado-Gómez *et al*., 2000; Müller *et al*., 2005; Rocha- Peña *et al*., 2002) but the cause-effect relationship had not been demonstrated in these reports.

The results of the present study support and extend the work of Salibe and Moreira, (1965) 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 within two years a significant number of small bark cracks. However, complete fulfillment of Koch's postulate

requires aging of these symptomatic lime trees and the development of the small cracks into characteristic "Quebra-galho" symptoms.

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Capítulo IV

Molecular and Biological characterization of natural variants of *Citrus dwarfing viroid*

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Abstract

Citrus dwarfing viroid (CDVd) is a member of the genus *Apscaviroid* belonging to the family *Pospiviroidae.* CDVd has been proposed as a dwarfing agent to control tree size in high-density plantations. Several types of CDVd variants (CVd-IIIa, CVd-IIIb and CVd-IIIc) with sizes of 297, 294 and 291 nt, respectively, had been identified but the information regarding the effect of specific changes on its biological properties is very limited. The characterization of thirty-three field isolates of CDVd by SSCP, cloning and sequence analysis showed that they contained either homogeneous or heterogeneous populations of sequence variants. The most frequent sequence/s of each isolate were identified. Alignment and phylogenetic analysis of 34 variants with the reference sequences showed that 29.4%, 58.8% and 11.8% were related to CVd-IIIa, CVd-IIIb and CVd-IIIc respectively. Five distinct variants were selected for their biological characterization on Etrog citron. Symptom expression and plant growth data demonstrated that two variants acted as severe strains, two as moderate strains and one as a very mild strain. The major changes discriminating these variants were: (i) two deletions and an insertion that result in a reorganization of the base pairing of the terminal left loop found in two of the variants; (ii) two (G42→A and C52→U) changes found in one of the variants; and (ii) as many as thirteen changes located in the right and left regions flanking the CCR found in one of the variants. The implication of these changes in modulating symptom expression is discussed.

Introduction

The beneficial effects of high density planting in citrus orchards has been the object of many studies addressing such issues as improved early returns, reduced labor costs and efficient land use (reviewed by Hutton *et al*., 2000). However, its implementation requires an efficient control of tree size that can be achieved by different means. The pioneering work conducted in Australia lead to the identification of "graft-transmissible dwarfing factors" (Fraser *et al.,* 1961), some of which were associated with exocortis bark scaling whereas others were not. Following the discovery of *Citrus exocortis viroid* (CEVd) as the causal agent of the exocortis disease (Semancik *et al*., 1972; Vernière *et al*., 2004), additional viroids were identified (Duran-Vila *et al*., 1986, 1988) and subsequently found to induce different levels of stunting in field grown trees (Hadas *et al*., 1989; Hutton *et al*., 2000; Schinghamer and Broadbent, 1997; Semancik *et al*., 1997; Vernière *et al*., 2004). Among the viroids tested in long-term field assays, *Citrus viroid III* (CVd-III) recently renamed *Citrus dwarfing viroid* (CDVd) (www.ictvonline.org/virus taxonomy), has been recognized as the most promising viroid to control tree size without undesirable effects.

Viroids are small, single-stranded, circular RNAs that replicate autonomously when inoculated in their plant hosts. CDVd is a member of the genus *Apscaviroid* (type member *Apple scar skin viroid* – ASSVd) belonging to the family *Pospiviroidae* whose members are characterized by the presence of a "central conserved region" (CCR) and the absence of RNA self-cleavage mediated by hammerhead ribozymes (Flores *et al.*, 2005). Following the model proposed by Keese and Symons (1985), the rod-like secondary structure of members of the family *Pospiviroidae* is divided into five structural-functional domains: P (pathogenicity), C (central), V (variable), T_L (terminal left) and T_R (terminal right). However, in members of the genus *Apscaviroid*, all restricted to woody species, studies to understand the relationship between different portions of the RNA secondary structure and the biological properties are scarce and whereas the CCR and the "terminal conserved region" (TCR) have been clearly identified, the presence and role of the P, V, T_L and T_R domains remains unknown.

CDVd, like the other members of the genus *Apscaviroid* has a rod-like secondary structure and possesses a CCR (Koltunow and Rezaian, 1989) and a TCR species-characteristic of the genus *Apscaviroid.* The host range of CDVd appears to be restricted to citrus and citrus relatives, and no specific symptoms have been associated with CDVd infection in commercial species used as rootstocks or as cultivars with the possible exception of finger imprint and bark striation sometimes observed on the trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock and associated with sprinkler irrigation (Vidalakis *et al.*, 2004). In the Etrog citron (*Citrus medica* L.) indicator, CDVd induces mild stunting and a "leaf dropping pattern" due to a moderate epinasty resulting from petiole and mid-vein necrosis.

Several types of CDVd variants were initially recognized by their distinct mobilities in sequential polyacrylamide gel electrophoresis (sPAGE) (Duran-Vila *et al.*, 1988). These types of variants were latter characterized as three distinct sequences (CVd-IIIa, CVd-IIIb and CVd-IIIc) with sizes of 297, 294 and 291 nt, respectively (Rakowsky *et al*, 1994; Stasys *et al.,* 1995; Semancik *et al.*, 1997). These three sequences differ in as much as 18 nucleotides located in the left and right regions flanking the CCR, but limited information is available regarding whether or not these changes are associated with distinct biological properties. In addition, since CDVd propagates in its hosts as populations of closely related sequence variants (Owens *et al.,* 1999), some minor sequence variants affecting certain regions of the molecule can be easily overlooked.

The objective of the present work is the identification of additional sequence variants and the characterization of their biological properties.

Materials and methods

Viroid sources

From a collection of field isolates recovered from different hosts and different locations, 33 were selected to perform the present study (Table 1).

Table 1. Selected of field isolates of CDVd.

Sample	Geographic origin	Source species	GeneBank accession[a]	
1	IVIA - Valencia (Spain)	'Navelina' sweet orange	EU 934007	
2	IVIA - Valencia (Spain)	'Navelina' sweet orange	EU 934009	
3	IVIA - Valencia (Spain)	'Thompson navel' sweet orange	EU 934013	
4	IVIA - Valencia (Spain)	'Washington navel' sweet orange	EU 934028	
5	IVIA - Valencia (Spain)	'Barberina' sweet orange	EU 934003	
6	IVIA - Valencia (Spain)	'Barberina' sweet orange	EU 934025	
7	IVIA - Valencia (Spain)	'Peret' sweet orage	EU 934024	
8	IVIA - Valencia (Spain)	'Newhall' sweet orange	EU 934015	
9	IVIA - Valencia (Spain)	'Nules' clementine	EU 934014	
10	IVIA - Valencia (Spain)	'Nules' clementine	EU 934022	
11	IVIA - Valencia (Spain)	Clementine (late)	EU 934020	
12	IVIA - Valencia (Spain)	Rough lemon (333M)	EU 934010	
13	IVIA - Valencia (Spain)	'Seminole' tangelo	EU 934005	
14	IVIA - Valencia (Spain)	'Frost' satsuma	EU 934012	
15	IVIA - Valencia (Spain)	'Oroval' grapefruit	EU 934016	
16	IVIA - Valencia (Spain)	Rangpur lime (334M),	EU 934023	
17	IVIA - Valencia (Spain)	'Nules' clementine (E117)	EU 934004	EU 934018
18	IVIA - Valencia (Spain)	'Verna' lemon	EU 934032	
19	Murcia (Spain)	'Local' lemon	EU 934008	
20	Murcia (Spain)	'Local' lemon	EU 934011	
21	Murcia (Spain)	'Local' lemon	EU 934030	
22	Murcia (Spain)	'Local' lemon	EU 934027	
23	Murcia (Spain)	'Local 'lemon'	EU 934029	
24	UCR, California (USA)[b]	'Eureka' lemon	EU 934000	
25	UCR, California, USA)[b]	Isolate E822	EU 934031	
26	Sicily (Italy)[c]	'Comune' clementine	EU 934026	
27	Bebedouro - SP (Brazil)[d]	'Baianinha' sweet orange	EU 934002	
28	Bebedouro - SP (Brazil)[d]	'Hamlin' sweet orange	EU 934017	
29	Bebedouro - SP (Brazil)[d]	'Hamlin' sweet orange	EU 933999	
30	Bebedouro - SP (Brazil)[d]	'Hamlin' sweet orange	EU 934006	
31	Tanuf (Sultanate of Oman)[e]	'Baladi' sweet orange	EU 934001	
32	Sohar (Sultanate of Oman)[e]	'Valencia' sweet orange	EU 934019	
33	Sohar (Sultanate of Oman)[e]	'Washington navel' sweet orange	EU 934021	

[a]Genebank accession number of the most representative sequence variant/s of each isolate. Sequence variants selected for infectivity assays are shown shaded.
[b]Kindly provided by J.S. Semancik (Plant Pathology Department, University of California, Riverside, USA).
[c]Kindly provided by S. Davino (Dipartimento di Scienze e Tecnologie Fitosanitarie, Universita degli Studi di Catania, Catania, Italy)
[d]Kindly provided by E. Sanches Stuchi (Estaçao Experimental de Citricultura, Bebedouro – SP, Brazil).
[e]Kindly provided by J.M. Bové (INRA and Université de Bordeaux 2, France), Y.M. Al-Raeesy (Crop Protection Research Center, Seeb, Sultanate of Oman) and H. Dietz (Directorate General of Agricultural and Veterinary Services, Royal Court Affairs, Sohar, Sultanate of Oman).

All these isolates had been graft-inoculated on the sensitive selection 861-S1 of Etrog citron grafted on Rough lemon rootstock and shown to contain RNAs with the mobility of CDVd by sPAGE analysis.

cDNA synthesis, PCR amplification and cloning

First-strand cDNA was synthesized in a final volume of 20 μl with 15 U of the reverse transcriptase ThermoScript^TM Rnase H^- (ThermoScript-RT, Invitrogen®) using the CDVd-specific reverse primer CVd-III-RT (0.75 μM), as described by Bernad and Duran-Vila (2006). Second-strand synthesis and PCR amplification (final 50 μl volume) was performed using 4 μl of the first strand cDNA reaction mixture, 1 U of *Taq* DNA and the reverse and forward primers (CVd-III-R1 and CVd-III-F1, respectively) (0.5 μM each) (Bernad and Duran-Vila, 2006) and dNTPs (0.12 mM each) in buffer containing (Tris-HCl 10 mM, pH 9.0, KCl 50 mM; MgCl$_2$ 1 mM). Primer-directed selection was minimised as both primers correspond to a conserved sequence in the upper strand of the CCR. The cycling profile consisted of a denaturation step at 95°C for 5 min, followed by 30 cycles (94°C for 30 s, 60°C for 30 s and 72°C for 2 min) and an extension step at 72° C for 5 min. Electrophoresis in 2% agarose gels confirmed the synthesis of products of the expected size.

For cloning purposes, the DNA bands were excised from the agarose gel and purified with the "GFX^TM PCR–DNA and Gel Band Purification Kit" (Amersham Bioscience). The purified DNA was ligated in the pGEM-T vector (Promega), and the recombinant plasmids were used to transform DH5α *E. coli* cells. Plasmids from transformed cell cultures were purified with the Perfectprep® Plasmid Mini Kit, and the presence of the desired inserts was confirmed by PCR using primers CVd-III-R1 and CVd-III-F1.

Single- strand conformation polymorphism (SSCP) analysis

Aliquots (3 μl) of the amplified viroid-cDNA inserts were mixed with 2 μl of denaturing solution (90% formamide, 25 mM EDTA [pH 7], 0.05% xylene cyanol and 0.05% bromophenol blue), heated at 95°C for 10 min and cooled immediately on ice. The partially denatured DNA strands were separated by PAGE in 14% gels (14×11.5×0.075 cm) containing 5% glycerol in TBE buffer (Tris-Borate 89 mM; EDTA 2 mM, pH 8) at 200 V for 16 h at 4ºC and visualized by silver staining (Igloi, 1983). Under these electrophoretic conditions the DNA migrates as two partially-denatured single strands (hDNA and cDNA) homologous and complementary to the viroid sequence, respectively (Palacio and Duran-Vila, 1999).

Sequencing and sequence analysis

RT-PCR amplicons or cloned viroid-cDNAs were sequenced with an ABI PRISM 377 apparatus (Perkin Elmer). Alignment of multiple sequences was performed using the program Clustal W (Thompson *et al.,* 1994). Phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei, 1987) based on 10.000 replicates and the consensus trees were condensed at the 80% bootstrap value level. Genetic distances were calculated following the method of Jukes and Cantor (1969) after manual adjustment for maximizing sequence similarities. All these analysis were conducted using the MEGA 3.1 program (Kumar *et al.,* 2004). Viroid secondary structures were obtained with the MFOLD program (circular version) from the GCG package (Zuker *et al*., 1999) and with RNAviz program (De Rijk and De Wachter, 1997).

Infectivity assays

Monomeric viroid-DNA inserts were recovered as blunt-end PCR products using primers (0.5 µM each) CVd-III–R1 and CVd-III–F1 phosphorylated at the 5' end and 1 U *Pfu* DNA polymerase (Stratagene) that generate blunt end products. The reaction was performed in PCR buffer (Tris-HCl 10 mM, pH 9,0, KCl 50 mM; MgCl$_2$ 1 mM), dNTPs (0.12 mM each) and the same cycling profile described above. DNA synthesis was confirmed by electrophoresis in 2% agarose gels and the bands eluted from the gel were subjected to ligation with 1 U of T4 DNA ligase (Gibco) at room temperature for 20 min. The dimeric molecules were cloned in pBluescript II KS (+) digested with *Eco*RV and the plasmids from transformed cells were sequenced to verify the desired head-to-tail orientation of the dimeric inserts. Clones with these inserts were linearized with *Hind* III or with *Eco*RI depending on the orientation of the insert and used as a template in a transcription reaction with 1 mM NTPs, 1 mM DTT and 50 U of T3 or T7 RNA polymerase, depending on the orientation of the dimeric insert, to produce dimeric transcripts homologous to the viroid sequence. Three Etrog citron seedlings were slash-inoculated (50 ng of transcript per plant) and kept in the greenhouse at 28º-32ºC. Infection was assessed by northern hybridization (Murcia *et al*., 2009) and confirmed by RT-PCR (Bernad and Duran-Vila, 2006).

Northern hybridization

Tissue samples (5 g) were powdered in liquid nitrogen and homogenized in 5 ml of extraction buffer (0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7.0; 4% (v/v) 2-mercaptoethanol) and 15 ml of water-saturated phenol. The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 µl of TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM MgCl$_2$ pH 7.4) (Semancik *et*

al., 1975). The RNAs separated in 5% PAGE under non-denaturing conditions (Morris and Wright, 1975) or in sPAGE (Rivera-Bustamante *et al.*, 1986) were electroblotted (400 mA for 1 h) from the gel to positively charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) and immobilized by UV cross-linking. DIG-labeled CDVd-specific probes were synthesized by PCR as described by Palacio *et al.*, (2000). Prehybridization (at 42ºC for 2-4 h) and hybridization (at 60ºC overnight) were performed in 50% formamide and 5×SSC buffer (SSC: 150 mM NaCl; 15 mM sodium citrate; pH 7.0) containing 0.02% SDS, 0.1% N-laurylsarcosine and 2% blocking solution (Roche). After hybridization the membranes were washed twice in 2×SSC, 0.1% SDS at room temperature for 15 min, and once in 0.1×SSC, 0.1% SDS at 60ºC for 60 min and revealed by autoradiography with an anti-DIG alkaline phosphatase conjugate and the chemiluminiscence substrate CSPD (Roche).

Symptom evaluation and statistical analysis

Swingle citrumelo seedlings were graft inoculated 15 cm above the soil level with two bark chips from infected citron seedlings. Infection was confirmed by northern hybridization and RT-PCR. Once infection was assessed, a bud of the selection 861-S1 of Etrog citron was propagated on each seedling immediately above the inoculum site and allowed to grow as a single shoot for five months at 28-32ºC for symptom evaluation. After five months, the citron shoot was cut 20 cm above the grafting site and the growth was monitored weekly.

Citron height data were subjected to one-way ANOVA taking into consideration for each plant and treatment: (i) the average height values (mean of the measurements taken at weekly intervals), and (ii) the linear component that refers to the slope of the growth curve. In addition, orthogonal contrast (Bewick *et al.*, 2004) and the LSD intervals (95%) post-test was applied.

Results

Identification of CDVd variants

Nucleic acid extracts from Etrog citron plants graft-inoculated each with one of 33 field sources of CDVd collected in different citrus growing areas (Table 1) were subjected to RT-PCR using CDVd-specific primers. In all instances, amplified DNAs with the mobility expected for CDVd were synthesized (data not shown). SSCP analysis of the DNA amplicons revealed differences in the migration of the single-strand DNAs (ssDNAs) indicating the existence of variations in their conformation and, therefore, in their nucleotide sequences (some examples are shown in Fig. 1A).
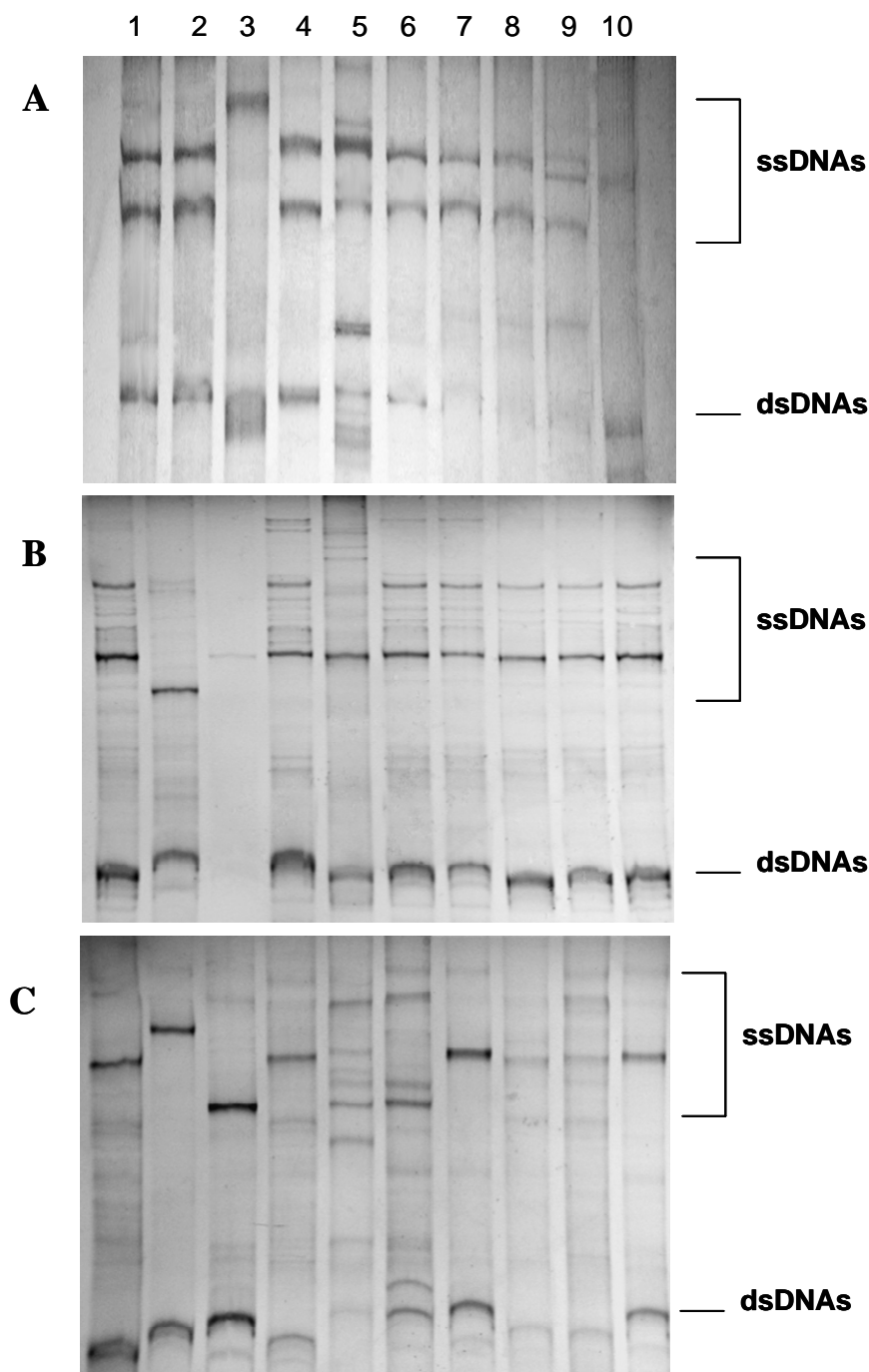
Fig. 1 (A) SSCP analysis of the DNA amplicons generated by RT-PCR using CDVd specific primers. Lanes 1 to 10 correspond to DNA amplicons obtained from 10 samples (See sample numbers in Table 1). **(B)** SSCP analysis of the inserts of ten clones recovered from a CDVd isolate of a 'local' lemon (sample 21) that contains a homogenous population of variants. **(C)** SSCP analysis of the inserts of ten clones from a CDVd isolate recovered from a 'Nules' clementine (sample 9) that contains an heterogeneous population of variants.

The DNA recovered from each field isolate was ligated to a cloning vector to recover clones containing full-length viroid-DNA inserts. For each cloning assay, the inserts of at least ten clones recovered by PCR were subjected to SSCP analysis. Under the conditions of

denaturation and electrophoresis used, the partially denatured double stranded DNAs (dsDNAs) migrated as two or more ssDNAs, hDNA and cDNA, respectively homologous and complementary to the viroid sequence. Since the migration of the hDNA and cDNA strands depends on nucleotide sequence and therefore on their conformation, the different SSCP profiles indicated the existence of different sequence variants.

The electrophoretic profiles obtained provided an indication of the heterogeneity of the different CDVd isolates. In some isolates a most frequent SSCP profile was clearly identified and found to be identical to that of the source DNA used for cloning (an example is shown in Fig. 1B), indicating that the isolate contained a homogeneous population of sequence variants.

In other isolates a dominant profile could not be identified (an example is shown in Fig. 1C), indicating that the isolate contained an heterogeneous population of sequence variants. From each isolate, at least three clones were selected for sequencing, and a sequence was considered to be the most representative of the isolate when it was found in at least two independent clones. In 32 of the 33 isolates tested, a single sequence variant was identified as the most frequent of each isolate, whereas in the case of isolate E117 recovered from a 'Nules' clementine tree (IVIA- Valencia, Spain) two sequence variants were selected as being equally representative (Table 1, sample 17). The nucleotide sequences of the 34 variants are now available in the Genebank (see accession numbers in Table 1)

Comparison with other natural variants of CDVd

From the sequence variants of CDVd available (Subviral RNA DATAbase and NCBI-Gen Bank) at the time when the present study was initiated, those recovered from natural sources (variants generated by induced mutation were not considered) were aligned to determine sequence identities. Thirty-three distinct variants excluding those that had already been extensively characterized by Owens *et al*., (2000) were selected and the Neighbor-joining phylogenetic tree produced by the MEGA 3.1 program (10000 replications of bootstrap test) showed that 20 were related to the CVd-IIIb variant (AF184147) (Rakowski *et al*., 1994), six to the CVd-IIIa variant (S76452) (Rakowski *et al*., 1994), and six to the CVd-IIIc variant (AF184149) (Semancik *et al*., 1997) (Fig. 2).
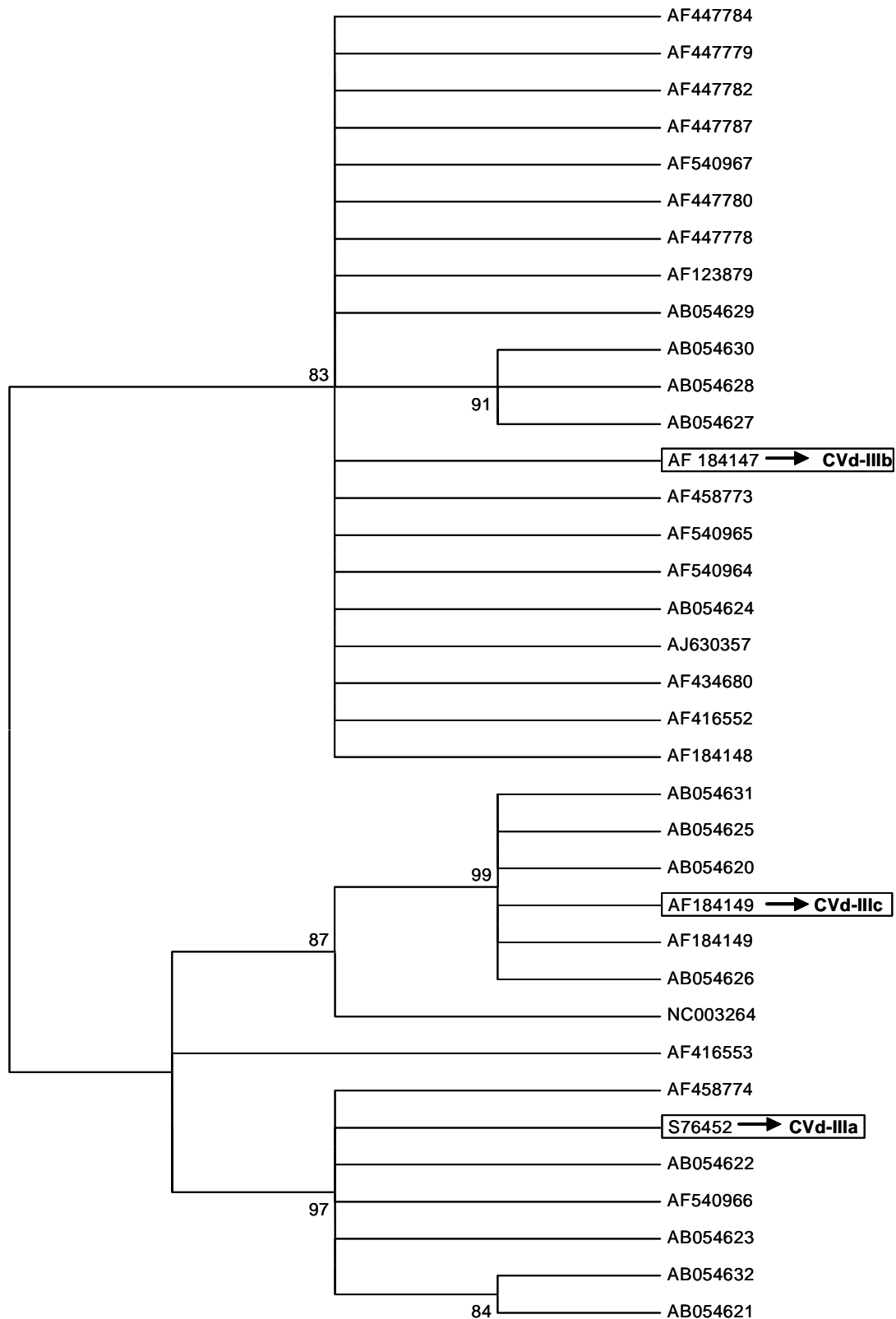
Fig. 2 Condensed Neighbor-Joining phylogenetic tree at the 80% bootstrap value level for the 33 CDVd sequences retrieved from viroid databases. Nucleotide distances were estimated using the Jukes and Cantor method (Jukes and Cantor, 1969). Booststrap values (%) based in 10.000 replicates are indicated in the nodes. Reference sequences are shown boxed.

Sequence alignment of the 34 sequences generated in the present study with the three reference variants (CVd-IIIa, CVd-IIIb and CVd-IIIc) of CDVd revealed that ten (29.4%) were closely related to CVd-IIIa with identities ranging from 96.0 to 99.7%, twenty (58.8%) were closely related to CVd-IIIb with identities ranging from 98.0 to 100%, and one was identical to CVd-IIIc (Table 2). The remaining three variants presented similar identities (Table 2, samples 11, 32 and 33) with CVd-IIIa, CVd-IIIb and CVd-IIIc. These observations were in accordance with the Neighbor-joining phylogenetic tree obtained with these 34 variants and the reference sequences of CVd-IIIa, CVd-IIIb and CVd-IIIc (Fig. 3).

Based on the above results five sequence variants were selected for their biological characterization: (i) EU934026 (referred as CDVd$^{IIIa\ it}$) that had been recovered from a 'Comune' clementine tree from Sicily (Italy) and differed in a single change from the reference sequence of variant CVd-IIIa (Table 2); (ii) EU934004 and EU934018 (referred as CDVd^{IIIb} and CDVd$^{IIIb\ sp}$, respectively) that had been recovered from a single 'Nules' clementine tree from Spain. CDVd^{IIIb} was identical to the reference sequence of CVd-IIIb and CDVd$^{IIIb\ sp}$ differed in three changes (Table 2) that affected the organization of the secondary structure of the T_L domain (Fig. 4); (iii) EU934022 (referred as CDVd^{IIIc}) that had been recovered from a 'Nules' clementine tree from Spain and was identical to the reference sequence of variant CVd-IIIc; and (iv) EU934019 (referred as CDVd^{so}) recovered from a 'Valencia' sweet orange from Sohar (Sultanate of Oman) presented numerous changes with the closest variant CVd-IIIa (Table 2). Some of these changes affected the conformation of the T_L domain that presented the same organization as CDVd$^{IIIb\ sp}$. The primary and predicted rod-like secondary structures of these variants are shown in Fig. 4.

Infectivity and viroid titers

To obtain infectious preparations of the five selected variants, head-to-tail dimeric cDNAs were synthesized and used as templates to produce the corresponding *in vitro* transcripts that were mechanically inoculated to three citron seedlings each. Fourteen months after inoculation, analysis by northern-blot hybridization confirmed infection of some of the inoculated citrons (data not shown). These results were confirmed by RT-PCR, and amplicon sequencing showed that in all instances the sequences were identical to those of the variants inoculated. With the exception of the only plant that became infected with CDVd^{so}, at an unusually low viroid titer, the viroid concentrations of all the infected citron plants were high and comparable to those of the graft-inoculated controls (data not shown).
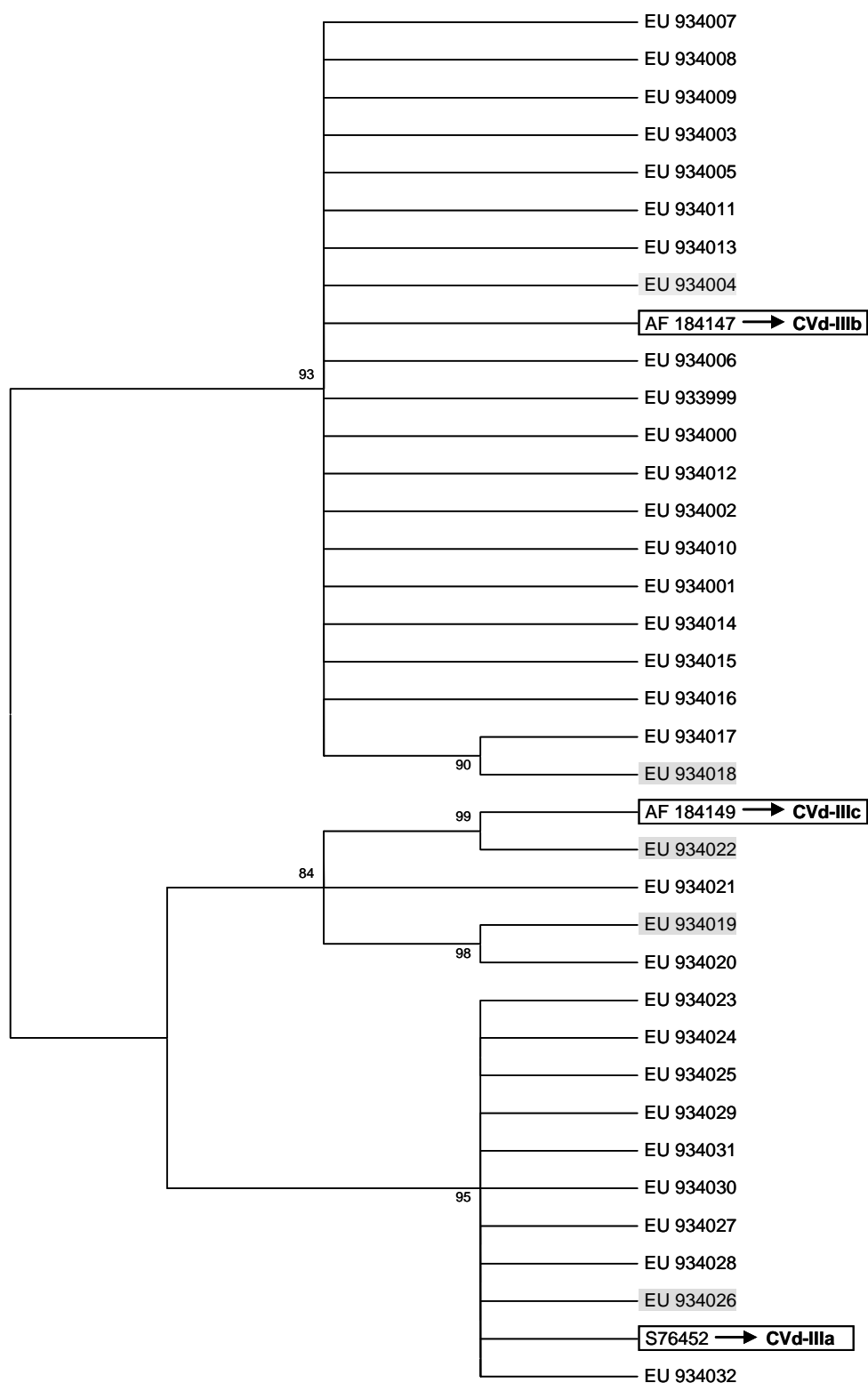
Fig. 3. Condensed Neighbor-Joining (Morris and Wright, 1975) phylogenetic tree (80% bootstrap value level) of 34 variants and the three CDVd reference sequences (CVd-IIIa, CVd-IIIb an CVd-IIIc). Nucleotide distances were estimated using the Jukes and Cantor method (1969) after manual adjustment to maximize sequence similarities. These analyses were conducted with the MEGA 3.1 program (Kumar *et al.*, 2004). Boostrap values (%) based in 10.000 replicates are indicated in the nodes.

Table 2 Molecular characteristics of the predominant sequence variants identified in thirty three field isolates of CDVd.

| Sample[a] | GeneBank accession | Size (nt) | Identity (%)[b] | | | Specific changes from the closest reference variant |
			CVd-IIIa	CVd-IIIb	CVd-IIIc	
4	EU934028	297	99.7	96.0	93.8	A113→C
21	EU934030	297	99.6	96.0	93.8	A113→C
22	EU934027	297	99.6	96.0	93.8	A113→C
23	EU934029	297	99.7	96.0	93.8	A113→C
25	EU934031	297	99.7	96.0	93.8	A113→C
26[c]	EU934026	297	99.7	96.0	93.8	A113→C
7	EU934024	297	99.3	95.6	93.5	A113→C, A163→U
18	EU934032	297	99.0	95.2	93.1	A53→U, A113→C, C245→U
6	EU934025	297	98.6	95.0	92.7	A113→C, G137→A, U138 →C, A163→U
16	EU934023	297	96.0	93.5	91.4	C36→U, U39→C, U40→G, G65→A, A113 →C, A163→U, C228→U, U251→C, A252→U, A254→C, A255→G, G258→A
1	EU934007	294	96.6	100.0	94.5	
2	EU934009	294	96.6	100.0	94.5	
3	EU934013	294	96.6	100.0	94.5	
5	EU934003	294	96.6	100.0	94.5	
12	EU934010	294	96.6	100.0	94.5	
13	EU934005	294	96.6	100.0	94.5	
14	EU934012	294	96.6	100.0	94.5	
17[c]	EU934004	294	96.6	100.0	94.5	

27	EU934002	294	96.6	100.0	94.5	
30	EU934006	294	96.6	100.0	94.5	
31	EU934001	294	96.6	100.0	94.5	
9	EU934014	294	96.0	99.3	93.8	G111→U, U138→C
19	EU934008	294	96.3	99.7	94.1	C30→U
20	EU934011	294	96.3	99.7	94.1	G250→U
24	EU934000	294	99.3	99.7	94.1	A67→G
29	EU933999	294	96.3	99.7	94.1	A67→G
17[c]	EU934018	293	96.0	99.3	93.8	3→-G, 4→-G 288→+U
28	EU934017	293	96.0	99.3	93.8	3→-G, 4→-G 288→+U
8	EU934015	294	96.3	99.6	94.1	U138→C
15	EU934016	294	94.6	98.0	92.4	3→-G, 4→-G, 41→+U, G135→A, U136→C, A161→U, 289→+U
10[c]	EU934022	291	95.6	95.5	100.0	
33	EU934021	293	96.6	96.0	96.7	68→+A, C144→A, 173A→U, 182→+A, 206C→U, 207A→U, 209G→A, 212A→G, U221→C, C223→A
11	EU 934020	293	95.6	94.8	95.2	3→-G, 4→-G, 54U→C 65G→A, A113 →G, 173U→ A, 184→-C, 185 →-A, 208A→U, 209A→U, 211U→A, 213→ -A, U215→A, C228→U, 293→-U
32[c]	EU934019	293	94.5	93.5	93.8	3→-G, 4→-G, G42→A, G63→A, A 111 →C, U136→C, U171→A, 182→-C, 183→-A, A197 →C, 203A →U, 204A→U, 206U→A, 208U→-A, 210U→A,

[a]Sample number as shown in table 1.
[b]Sequence identities with the closest reference variant. Gene bank accession CVd-IIIa variant (S76452), CVd-IIIb variant (AF184147), CVd-IIIc variant (AF184149)
[c]Sequence variants selected for infectivity assays are shown shaded.
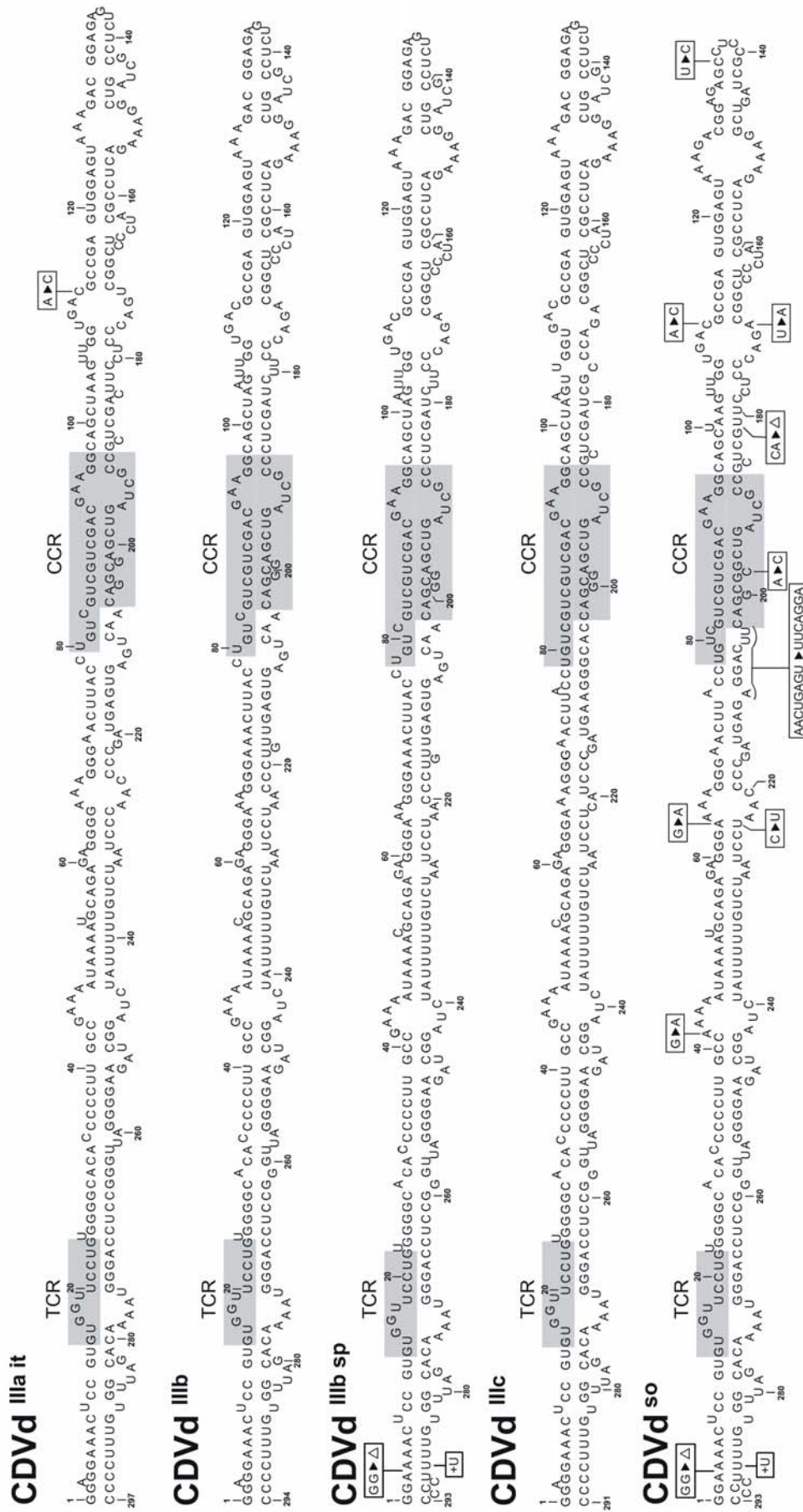
Fig. 4 Primary and predicted secondary structure obtained with the MFOLD program (circular version) from the GCG package (Zuker *et al.*, 1999), and with RNAviz program (De Rijk and De Watcher, 1997) of five sequence variants of CDVd showing the changes identified when compared with the closest reference sequence:CDVd[IIIa it] and CDVd[so] were compared with CVd-IIIa (Owens *et al.*, 2002). CDVd[IIIb] and CDVd[IIIb sp] were compared with CVd-IIIb; and CDVd[IIIc] was compared with CVd-IIIc. The conserved CCR and TCR regions are shown shaded.

To compare the performance of plants infected with these five variants of CDVd, five sets of three Swingle citrumelo seedlings were graft inoculated using as a source of inoculum two bark patches from the infected citron seedlings. Three additional non-inoculated Swingle citrumelo seedlings were kept as negative controls. Analysis by northern-blot hybridization and RT-PCR confirmed infection four months after inoculation. As found in the Etrog citron seedlings, the northern-blot hybridization results indicated that the titer of variant CDVd^{so} was considerably lower than those of the other variants (Fig. 5A). Amplicon sequencing revealed that the sequences of CDVdIIIa^{it}, CDVdIIIb and CDVd^{so} remained stable in the infected citrumelo seedlings, whereas CDVd$^{IIIb\ sp}$ presented two changes (A221→C, T223→A) and CDVd^{IIIc} one change (A254→T).
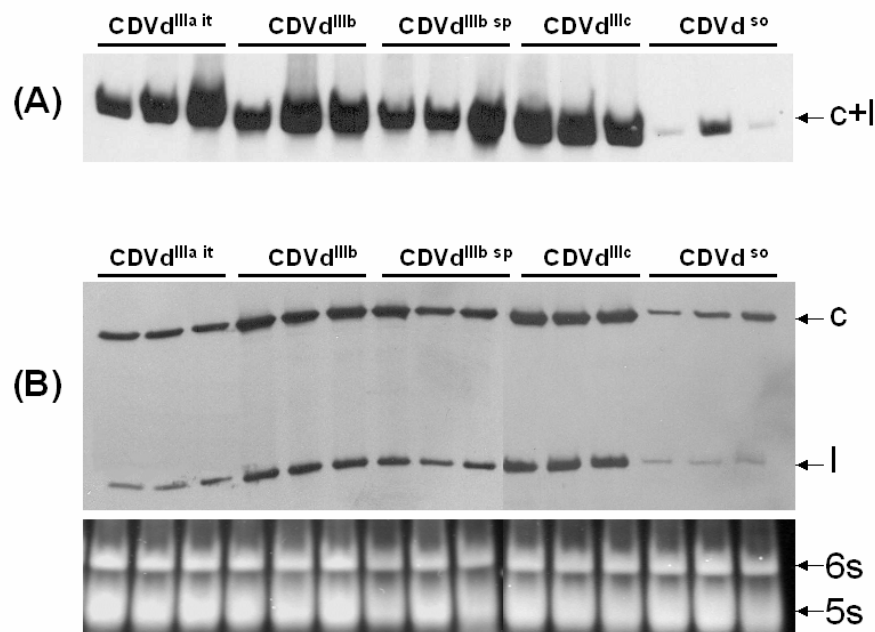
Fig. 5 Northern hybridization analysis of samples from Swingle citrumelo seedlings inoculated with five CDVd variants **(A)** and from the Etrog citron scion graft-propagated on the infected Swingle citrumelo seedlings **(B)**. **(A)** Samples were subjected to non-denaturing PAGE, electroblotted to membranes and hybridized with CDVd-specific probe. (c+l) point at the circular (c) and linear (l) forms of CDVd that run as a single band in non-denaturing PAGE. **(B)** Samples were subjected to sPAGE and the nucleic acids separated in the second denaturing PAGE were electroblotted to membranes and hybridized with CDVd-specific probe. (c) and (l) point at the circular and linear forms of CDVd that present different movilities in denaturing PAGE. Ethidium bromide staining of the 6S and 5S rRNAs viewed in the first non-denaturing gel of sPAGE showing that RNA levels in all preparations were comparable is shown below.

After four additional months, these citrumelo seedlings were used as rootstocks to graft propagate buds of the sensitive selection 861-S1 of Etrog citron. One month after grafting the citron buds started growing and they were subsequently trained to grow as a single shoot. Analysis of the fifteen Etrog citron plants by northern-blot hybridization showed that, similar to the situation with the citrumelo seedlings, the titer of variant CDVd^so remained considerably lower than those of the other variants (Fig. 5B). The Etrog citron plants were also subjected to RT-PCR, and the DNA recovered from each plant was ligated to a cloning vector to recover clones containing full-length CDVd-DNA inserts. Sequence analysis of three clones from each plant showed that CDVd^{IIIa it}, CDVd^{IIIb} and CDVd^so remained stable, whereas the changes identified in the citrumelo seedlings infected with CDVd^{IIIb sp} and CDVd^{IIIc} reverted to the sequence inoculated to the original citron seedlings.

Symptom expression and plant growth

The young leaves of the infected Etrog citron plants showed epinasty that was very prominent in plants infected with CDVd^{IIIa it} or CDVd^{IIIb sp} (Fig. 6A, 6B), mild in plants infected CDVd^{IIIb} and CDVd^{IIIc so} (Fig. 6C, 6D) and almost imperceptible in plants infected with CDVd^{IIIc} (Fig. 6E). In all instances, old leaves developed petiole necrosis that extended along the midvein of the leaves (Fig. 6G), causing the characteristic "leaf-dropping pattern". These symptoms were also severe in plants infected with CDVd^{IIIa it} or CDVd^{IIIb sp}, moderate in plants infected with CDVd^{IIIb} or CDVd^{IIIc so} and mild in plants infected with CDVd^{IIIc}. In addition, the plants infected with CDVd^{IIIa it} or CDVd^{IIIb sp} presented cracks that started at the basal end of the scion (Fig. 6H) and usually extended along the stem (Fig. 6I). These severe, moderate and mild symptoms were associated with different degrees of stunting that could not be adequately monitored because of irregular sprouting of the grafted buds.

After five months the plants were cut at the level of the second citron internode and the second flush of growth was measured weekly. Figure 7 displays data on citron growth over a thirteen-week period illustrating the differences found in growth patterns. Comparisons of the average height values and the linear components using one-way ANOVA test showed significant differences in the average height (P-value=0.014<0.05) and in the linear component that refers to the slope of the growth curve (P-value=0.021<0.05).

It should be noted that whereas the non-infected controls presented a smooth growth curve, the five CDVd-infected treatments appear to follow unusual patterns with periods of active growth alternating with periods of slow growth, and therefore reaching smaller sizes.
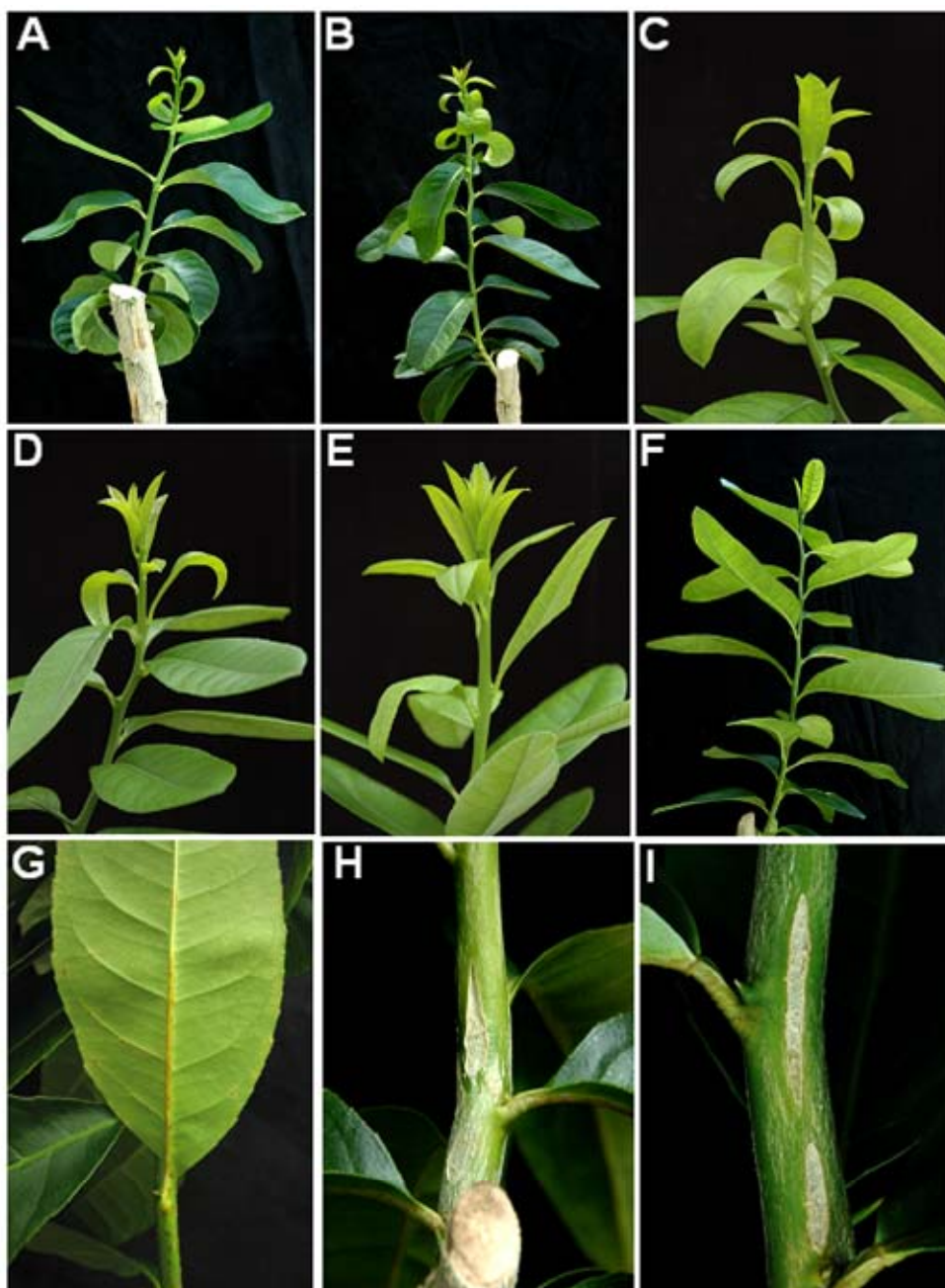
Fig. 6 Symptoms observed in Etrog citron plants infected with five CDVd variants. Epinasty on young leaves of Etrog citron infected with CDVd$^{IIIa\ it}$ (**A**), CDVd$^{IIIb\ sp}$ (**B**), CDVd^{IIIb} (**C**), CDVd^{so} (**D**) and CDVd^{IIIc} (**E**). Symptomless leaves of non-inoculated control (**F**). Petiole and midvein necrosis (**G**). Cracks at the basal end of the Etrog citron scion (**H**) usually extending along the stem (**I**) in plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$.

Orthogonal contrast confirmed that the average height (P-value=0.021<0.05) and the linear component (P-value=0.026<0.05) of CDVd-infected plants differed from the non-infected controls. Multiple range test by 95% LSD method indicated that the growth data (average height and linear component) clustered into three distinct groups: (i) plants infected with CDVd^{IIIc} that presented the mildest symptoms fell in the same group as the non-infected controls; (ii) plants infected with CDVd$^{IIIa\ it}$ or CDVd$^{IIIb\ sp}$ that presented the most severe

symptoms fell in the same group; (iii) plants infected with CDVd[IIIb] and CDVd[IIIc so] presented an intermediate position (Fig. 6 and 7).
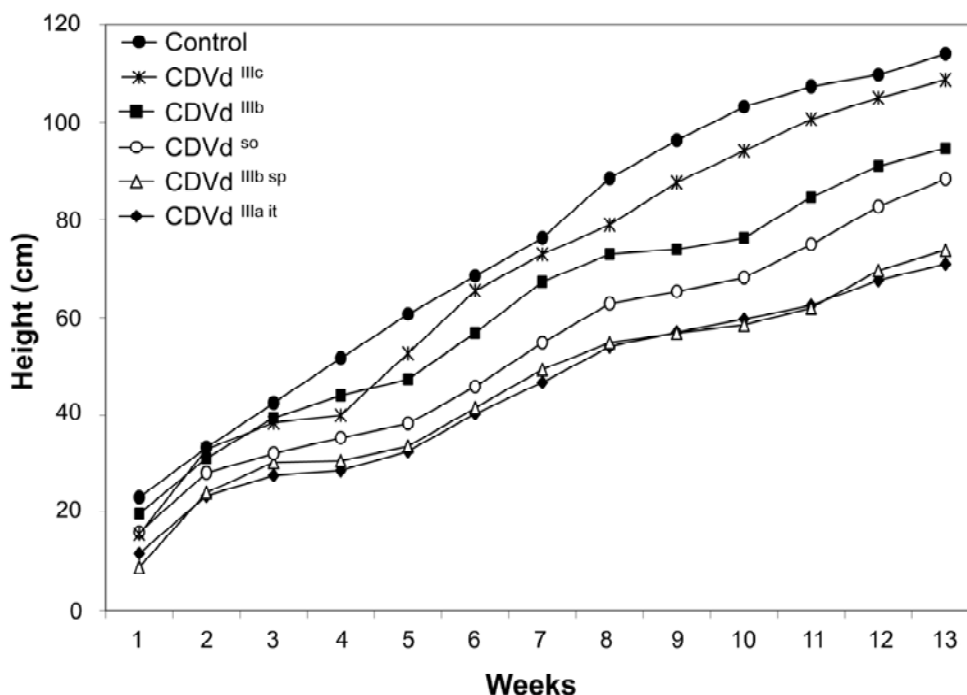
Fig. 7 Growth curves of the second flush of growth of Etrog citron plants infected with a five variants of CDVd. Data are the means of the height values determined over a thirteen-week interval.

Discussion

Long-term field assays revealed the potential of CDVd to control tree size in commercial plantings (Semancik *et al.*, 1997; Hutton *et al.*, 2000). However, in spite of the large number of sequence variants identified, limited information is available regarding the relationship between changes in the primary and secondary structures of CDVd and its biological properties.

Studies conducted by Owens *et al.,* (1999, 2000) showed that natural CDVd isolates propagate in their hosts as populations of closely related sequence variants with predominant variant/s representing a single or several fitness peaks. The results of the present study indicate that with the exception of a single CDVd source recovered from a clementine tree, all the others contained a clearly predominant variant.

The phylogenetic analysis performed with 71 additional variants available in databases and with the 34 variants generated in the present study confirmed the preponderance of CVd-IIIb and CVd-IIIb-related sequences, with fewer variants clustering around CVd-IIIa and CVd-IIIc. These results are in agreement with those generated with the molecular characterization and phylogenetic analysis of 86 sequence variants recovered from nine CDVd sources that

showed that the sequences of CV-IIIa and CVd-IIIb were predominant and that the variability observed was the result of both point mutation and RNA recombination (Owens *et al.,* 2000).

The strategy followed in the present study to select CDVd variants for their further biological characterization was designed to avoid non-infectious and/or reverting variants that may be present in the populations of the infected sources (Owens *et al.,* 1999). In addition, in order to avoid the selection of variants that had accumulated in a given host as a result of host driven selection (Bernad *et al*., 2005), Etrog citron plants infected by graft transmission from the original sources, were used as the source of tissue for the identification and selection of CDVd variants. The five variants selected were all predominant in the pool of variants present in the infected Etrog citron plants and all resulted infectious and stable in the citron seedlings that were initially inoculated with infectious transcripts. It should be pointed that after graft-transmission to Swingle citrumelo, three of the variants remained stable but the other two, CDVd^{IIIc} and CDVd$^{IIIb\ sp}$, presented one and two mutations, respectively. The accumulation of such mutations illustrate the host driven selection from the spectra of mutants generated in the absence of proofreading activity of the RNA polymerases involved in viroid replication (Gandía *et al*., 2005). However, as demonstrated in the case of CEVd (Bernad *et al*., unpublished), the mutations identified in citrumelo, reverted back to the original sequence, once the viroid reached and replicated in the Etrog citron scion.

The symptoms and growth of the infected Etrog citron plants indicate that the five variants studied behave as mild (CDVd^{IIIc}), moderate (CDVd^{IIIb} and CDVd^{so}) and severe strains (CDVd$^{IIIa\ it}$ and CDVd$^{IIIb\ sp}$) of CDVd. In addition, a good correlation was found between leaf and stem symptoms and the final size reached by the infected plants. It should be also noted that in contrast with the mild CDVd^{IIIc} strain that accumulated at high titers in infected plants, CDVd^{so} characterized as moderate strain, accumulated very low viroid titers. This observation demonstrates the lack of relationship between viroid accumulation and symptom expression.

Variants CDVd$^{IIIb\ sp}$ differ from CDVd^{IIIb} in only two deletions and an insertion that result in a reorganization of the base pairing of the terminal left loop. Inherent with these changes we found an increase in symptom severity, a result that demonstrates the involvement of the T_L domain in the pathogenicity of CDVd. The role of the T_L domain in modulating symptom expression had been previously demonstrated by infecting Etrog citron plants with a chimeric viroid constructed by exchanging the T_L domain of *Citrus viroid V* (CVd-V) by that of CDVd (Serra *et al*., 2009). Therefore, these results altogether should be considered as an evidence of the implication of the T_L domain in symptom expression not only of CDVd but also of other members of the genus *Apscaviroid.*

Variant CDVd[so] that behaved as a moderate strain differed in 2 deletions, 4 insertions and 13 changes from the closest variant, the mildest CDVd[IIIc] strain. Among these differences, CDVd[so] presents the two deletions and the insertion that affect the conformation of the terminal left loop and that was found to enhance the severity of the symptoms induced by CDVd[IIIb sp]. CDVd[so] also presents two changes, G42→A and C52→U, that are identical to those identified earlier and found to be associated with a reduction of symptom expression (Owens *et al.,* 1999; 2002)**.** The remaining changes mainly affected the right and left regions flanking the lower CCR stand and conferred to such regions a secondary structure very similar to that of the severe CDVd[IIIa it]. As a consequence of all those changes CDVd[so] should be considered as an unusual strain.

The results of many years of research on viroid dwarfing conducted in Australia using several cultivars of sweet orange grafted on different rootstocks showed that the dwarfing response was affected by the viroid/s inoculated, the inoculation time and the sensitivity of the rootstock to the inoculated viroid/s (Hutton *et al*., 2000). Additional dwarfing trials have focused on the potential of CDVd because it was not associated with undesired symptoms (Semancik *et al*., 1997; 2000; van Vuuren *et al*., 2002; Tessitori *et al*., 2002; 2005; Albanese *et al*., 1996). However, the long term dwarfing effect of CDVd on Moro sweet orange and clementine trees grafted on Troyer citrange and trifoliate orange growing in Sicily was found to be not as marked as anticipated (Polizzi *et al*., 1991; Albanesse *et al*., 1996). Therefore the choice of viroids and viroid strains as dwarfing agents must be adequately studied for specific rootstock/scion combinations and the specific growing conditions that are known to affect viroid replication and symptom expression.

The results of the present study demonstrate that strains with different levels of virulence exist. Although the symptoms induced in Etrog citron show a good correlation with their stunting properties, such effect should be further monitored before being used to control tree size in commercial rootstock/scion combinations.

Acknowledgments

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Capítulo V

Citrus viroids: Symptom expression and performance of Washington navel sweet orange trees grafted on Carrizo citrange

Abstract

Citrus are natural hosts of five viroid species: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd); *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd). CEVd and HSVd are the causal agents of the well known diseases of citrus, exocortis and cachexia. Other viroids have been found to induce specific symptoms and different degrees of stunting in trees grafted in trifoliate orange. A field assay was initiated in 1989 to establish the effect of each viroid on Washington navel sweet orange trees grafted on Carrizo citrange. Here we report the effect of viroid infection on symptom expression, tree size, fruit production and quality evaluated from 2004 to 2007. No bark scaling symptoms were observed in CEVd infected trees albeit they presented lesions and blisters in the roots. Viroid induced bark cracking symptoms were consistently found in CBCVd infected trees that were smaller and showed reduced yields. No major effects were found as a result of infection with CBCVd, HSVd or CDVd. Reduction in tree size was associated with small root systems. The quality of the fruits was not affected as result of viroid infection, except for the low caliber of the fruits harvested from HSVd infected trees.

Introduction

Citrus exocortis disease was described in 1948 as a bark shelling or scaling disorder affecting trees grown on the trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock (Fawcett and Klotz, 1948). Once graft transmission was demonstrated (Benton *et al.,* 1949; 1950), the disease was considered to be of viral etiology. Following the identification of Etrog citron (*Citrus medica* L.) as an exocortis sensitive host, biological indexing methods were developed (Calavan, 1968; Roistacher *et al*., 1977) and routinely used for detection purposes. With the discovery of viroids as a new class of plant pathogens (Diener 1971), the exocortis disease was demonstrated to be associated with the *Citrus exocortis viroid* (CEVd) (Semancik and Weathers, 1972).

The routine use of Etrog citron for indexing purposes revealed that field-grown plants induced a variety of symptoms ranging from severe to very mild which were erroneously considered for many years, as evidence for the existence of CEVd strains. Schlemmer *et al.* (1985) provided the first evidence indicating that viroids other than CEVd were responsible for the mild and moderate symptoms observed on inoculated citrons. Subsequently, with the development of a double electrophoresis system (sequential polyacrylamide gel electrophoresis, sPAGE), a number of circular RNAs with faster migration than CEVd were consistently identified in field isolates, and their viroid nature established by infectivity assays on Etrog citron (Duran-Vila *et al.,* 1986; 1988). These viroids initially termed "citrus viroids

(CVd)" were classified into five groups based on their electrophoretic migration in 5% sPAGE, sequence similarity determined by molecular hybridization against specific cDNA probes and host specific symptoms on the Etrog citron indicator (Duran-Vila *et al.,* 1988). Further sequencing confirmed that the proposed groups were consistent with the viroid species concept proposed by the International Committee on Taxonomy of Viruses (ICTV) (www.ictvonline.org/virusTaxonomy.asp). In addition to CEVd, *Citrus bent leaf viroid* (CBLVd) (former CVd-I), *Hop stunt viroid* (HSVd) (former CVd-II), *Citrus dwarfing viroid* (CDVd) (former CVd-III) and *Citrus bark cracking viroid* (CBCVd) (former CVd-IV) have been recognized as true viroid species. More recently two additional viroids, tentatively named *Citrus viroid OS* (CVd-OS) and *Citrus viroid V* (CVd-V) have been reported (Ito *et al.,* 2001; Serra *et al*., 2007).

The citrus cachexia disease, also known as xyloporosis, was first described in 1948 as discoloration, gumming and browning of phloem tissues, wood pitting and bark cracking on Orlando tangelo (*C. paradisi* Macf. × *C. reticulata* Blanco) (Childs, 1950) and suspected of being caused by a viroid (Roistacher, 1983). Characterization of citrus viroids other than CEVd lead to the identification of specific variants of HSVd, in which a specific 5/6-nucleotide motif located in the Variable (V) domain ("cachexia expression motif") was responsible for its pathogenicity, as the causal agent of the cachexia disease (Semancik *et al.,* 1988; Reawankaron and Semancik, 1988; Serra *et al*., 2008).

Even though viroids had been shown to induce different degrees of dwarfing in certain rootstock/scion combinations (Gillings *et al*., 1991; Semancik *et al*., 1997; Hutton *et al*., 2000), the effect of each viroid on field grown trees was not fully evaluated until 2004 when the results of a long term field assay in which clementine trees grafted on trifoliate orange rootstock inoculated with several viroid sources, were evaluated (Vernière *et al.,* 2004). Here we report the results of an assay conducted with Washington navel sweet orange (*C. sinensis* (L.) grafted on the exocortis sensitive Carrizo citrange (*P. trifoliata* × *C. sinensis*) in which the same viroid sources were used as a source of inoculum.

Materials and Methods

Viroid sources.

Several single viroid sources of CEVd, CBLVd, HSVd, CDVd and CBCVd were selected from the viroid collection maintained at Instituto Valenciano de Investigaciones Agrarias (IVIA). Each viroid source had been maintained in Etrog citron plants grafted on rough lemon that had been periodically tested to verify that they contained the expected single viroid source. Since this assay was initiated in 1989, only limited information was available

regarding the molecular characteristics and biological properties of the viroid sources selected. The information available now can be summarized as follows:

CEVd: Two isolates (CEVd-117 and CEVd-129) were chosen because they differed in the intensity of the symptoms induced in citron and *Gynura aurantiaca*. Further sequencing and biological characterization demonstrated that the sequences of these isolates (Gandía *et al.,* 2005; Chaffai *et al.,* 2007) presented in the Pathogencity (P) and Variable (V) domains the molecular characteristics of CEVd variants that had been classified as class A and class B (severe and mild, respectively) based on their pathogenicity on tomato (Visvader and Symons, 1986).

CBLVd: Two isolates (CVd-Ia and CVd-Ib), were initially selected because they differed in their electrophoretic mobility in sPAGE analysis. Isolate CVd-Ia had been recovered from a field source from Spain and it was highly homologous to the CBLVd (CVd-Ia) reported by Semancik *et al.,* (1997) but contained a set of nucleotide changes clustered in the left of the viroid secondary structure (Foissac and Duran-Vila, 2000). Isolate CVd-Ib (kindly provided by J.S. Semancik, University of California, Riverside) was similar to the type strain of CBLVd (Ben-Shaul *et al.,* 1995).

HSVd: Isolate CVd-IIa had been biologically characterized as a non cachexia inducing variant and sequencing demostrated that it lacked the "cachexia expression motif" in the V domain of the viroid secondary structure (Palacio-Bielsa *et al.,* 2004; Serra *et a.,* 2008). Isolates CVd-IIb and CVd-IIc had been characterized as cachexia inducing variants and sequencing demostrated that they contained the "cachexia expression motif" (Palacio-Bielsa *et al.,* 2004; Serra *et al.,* 2008).

CDVd: Isolates CVd-IIIa and CV-IIIb (kindly provided by J.S. Semancik, University of California, Riverside) were isolated from California and differed in 11 nucleotide changes (Rakowski *et al.,* 1994). CVd-IIIc and CVd-IIId were selected from Spain and characterized as distinct variants of CDVd (Murcia *et al.,* 2008).

CBCVd: The single isolate of CBCVd (kindly provided by J.S. Semancik, University of California, Riverside) was from California and it had been characterized to the type CBCVd strain (Francis *et al.,* 1995).

Plant materials and inoculation.

In June 1989, one-year-old Washington navel trees grafted on Carrizo citrange were graft-inoculated with one of 12 viroid isolates (six plants per viroid treatment and six non-inoculated controls). The following year the trees were transplanted in a randomized block

arrangement in a calcareous, alkaline (pH around 8.0), sandy-loam soil. The field plot was located at the Instituto Valenciano de Investigaciones Agrarias" (IVIA), Moncada (Valencia, Spain) on the East coast of Spain (Fig. 1A)

Fig. 1. Field plot of Washington navel trees grafted on Carrizo citrange graft-inoculated with twelve viroid isolates. **(A)** Aspect of the trees when the experiment was terminated. **(B)** Trunk from which the bark had been scrapped to see the presence of pitting and gumming symptoms. **(C)** Trees decapitated 50 cm above the bud union. **(D)** Trees in which the bark of the stumps had been partially removed for a final evaluation of symptoms on the bark and the wood above and below the bud union. **(E)** Stumps being pulled away from the soil. **(F)** Stumps from which the remaining trunk had been cut away. **(G)** Measuring the fresh weight of the root system.

The trees were subjected to the standard pruning and harvesting operations of the region. In order to shape the canopy of the trees, two major prunings were performed on 1991 and 1993, and suckers were removed in all subsequent years. Tools were disinfested with a sodium hypochlorite solution in between trees. In 2006-2007 all the trees were indexed to verify that they were actually infected and that the non-inoculated controls had remained viroid-free.

Viroid indexing.

Bark (5 g) stripped from young shoots was powdered in liquid nitrogen and homogenized in 5 ml of extraction buffer (0.4 M Tris-HCl pH 8.9; 1% (w/v) SDS; 5 mM EDTA pH 7.0; 4% (v/v) 2-mercaptoethanol) and 15 ml of water-saturated phenol. The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 µl of TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM MgCl$_2$ pH 7.4) (Semancik *et al.*, 1975). Aliquots of these nucleic acid preparations were analyzed by northern blot hybridization as described by Murcia *et al.,* (2009). Briefly, the RNAs separated in 5% PAGE (60 mA, 2 h) were electroblotted (400 mA for 1 h) from the gel to Nylon membranes, immobilized by UV cross-linking and hybridized with viroid-specific DIG-labelled DNA probes generated by PCR using as templates plasmids containing full-length viroid DNA inserts (Palacio *et al.*, 2000). Prehybridization and hybridization were performed in 50% formamide and the DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualized by autoradiography with the chemiluminiscence substrate CSPD (Roche).

Symptom evaluation.

Bark symptoms (scaling and cracking) were evaluated when the experiment was terminated in 2008. The bark was first scrapped above and below the budunion to verify the presence of pitting and gumming symptoms (Fig. 1B). The trees were decapitated 50 cm above the bud union (Fig. 1C) and the bark of the stumps was removed for a final evaluation of symptoms on the bark and the wood above and below the bud union (Fig. 1D). Finally the stumps were pulled out from the soil (Fig. 1E) for a final evaluation of symptoms in the roots. After cutting away the remaining trunk (Fig. 1F), the fresh weight of the root system was measured after and removing the soil that was attached to the roots (Fig. 1G).

Tree growth and fruit yield.

Tree height, trunk circumferences of the scion and the rootstock (10 cm above and below the bud line) were measured in 2004 and 2007. The canopy volume was estimated as Vc $= 4/3\pi R^3$ (R being the radius of the canopy calculated from the canopy height and two perpendicular width measurements). Fruits were harvested manually and fruit yield data were recorded in 2004, 2006 and 2007.

Fruit quality.

From October 2005 to February 2006, the evolution of the color and size (height and diameter) of ten randomly collected fruits from each tree was recorded at monthly intervals.

Color index was determined using a colorimeter (Minolta CR-300) (Jiménez-Cuesta *et al.,* 1981) taking into consideration the average of three independent measurements made at the equatorial zone of the fruit. During the same period measurements of fruit height and diameter were also recorded from the same sampled fruits.

External fruit quality parameters were determined using fruit samples (10 fruits per tree) collected from the 2007 harvest that were recorded as follows: (i) fruit shape determined as the average of the diameter/height values; (ii) average of rind thickness measured at the equatorial zone using a digital caliper; (iii) bulk weight of the ten fruits of each sample; (iv) bulk fruit size (volume) measured using the water displacement method; and (v) fruit density calculated as quotient between weight and fruit volume.

Internal fruit quality parameters were determined using fruit samples (10 fruits per tree) collected from the 2007 harvest that were recorded as follows: (i) volume of juice extracted from the ten fruit samples using a squeezer (Zumonat C40) that operates at 14 kg/cm^2 and contains a series of filters that retain the large particles; (ii) juice density measured with a densitometer (Proton 1000-1100) calibrated at 15ºC; (iii) soluble solids measured with a refractometer (Atago PR-101); (iv) total acidity measured by titration of 100 ml of juice with NaOH 0.1N and correcting the volume of NaOH (x 1.28) to obtain the corresponding percentage (%) of citric acid; and (v) maturity index calculated as the quotient of percentage of soluble solids and acidity percentage.

Statistical analysis.

The effect of viroid infection and the effect of viroid isolates was performed by repeated measures ANOVA. Comparisons with the control were done with the Dunnett test.

Results

Symptoms induced by viroid infection

No bark scaling symptoms were observed in any of the treatments (Table 1). Mild bark cracking symptoms were observed in several treatments, including the viroid-free controls. This mild bark cracking affected the rootstock as well as the scion (Fig. 2A). Severe bark cracking symptoms were consistently found in the rootstock of trees infected with CBCVd (6 of 6) (Table 1). As reported earlier in the case of trees grafted in trifoliate orange (Vernière *et al.,* 2004), severe cracking was more perceptible by the presence of characteristic green streaks easily visible after scrapping the bark (Fig. 2B).

Table 1. Symptoms observed on Washington Navel orange grafted on Carrizo citrange rootstock infected with single viroid sources.

Treatment		Bark scaling	Bark cracking		Pegs	Gummy Pits
Viroid	Isolate	No. trees*	Intensity	No. trees*	No. trees*	No. trees*
Control		0/6	Mild	6/6	0/6	0/6
CEVd	CEVd-117	0/6	-	0/6	1/6	0/6
	CEVd-129	0/6	Mild	6/6	1/6	1/6
CBLVd	CVd-Ia-117	0/6	-	0/6	0/6	3/6
	CVd-Ib	0/6	-	0/6	0/6	2/6
HSVd	CVD-IIa-117	0/6	-	0/6	1/6	3/6
	CVd-IIb	0/6	-	0/6	1/6	1/6
	CVd-IIc	0/6	Mild	1/6	0/6	2/6
CDVd	CVd-IIIa	0/6	Mild	2/6	1/6	2/6
	CVd-IIIb	0/6	-	0/6	0/6	0/6
	CVd-IIIc	0/6	-	0/6	0/6	1/6
	CVd-IIId	0/6	-	0/6	2/6	1/6
CBCVd	CVd-IV-Ca	0/6	Severe	6/6	0/6	0/6

 *Number of trees presenting symptoms over the total number of trees.
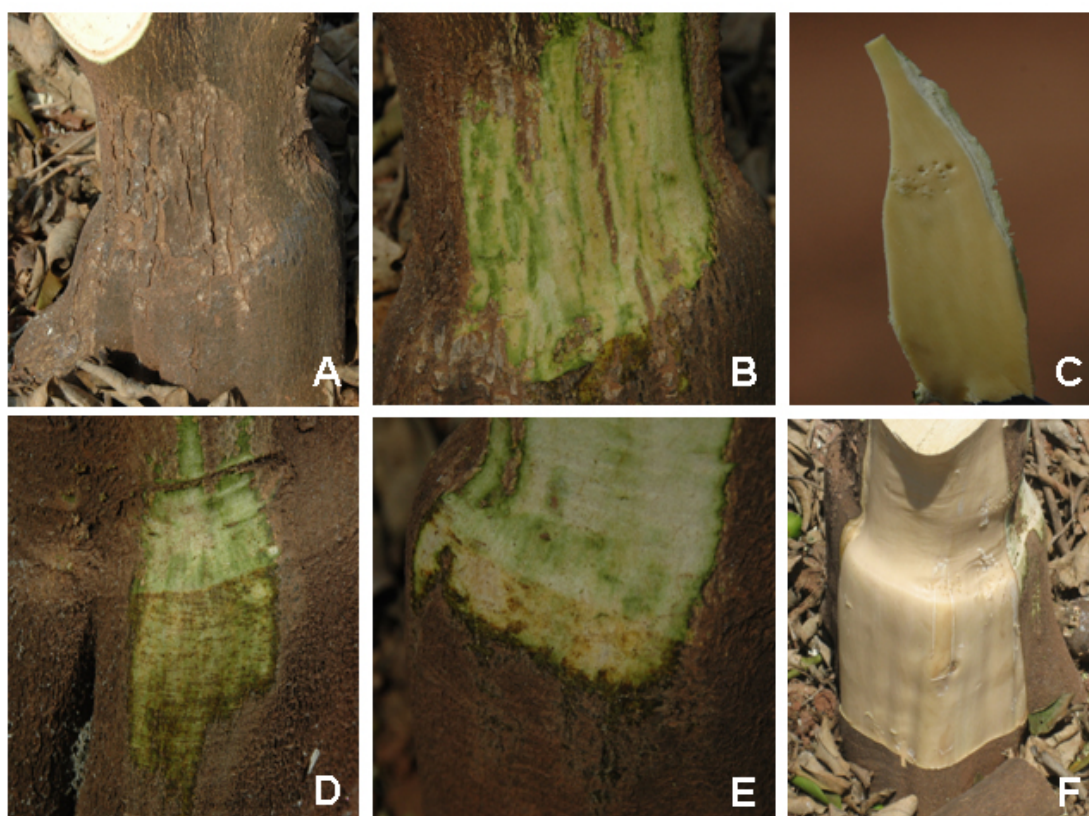
Fig. 2. (A) Unspecific bark cracking extending from the rootstock to the scion. **(B)** Green streaks observed in the wood after removing the bark. **(C)** Small pits in the cambial site of the bark. **(D)** and **(E)** Horizontal streaks of pits containing gum pockets. **(F)** Unaffected wood.

After removing the bark, small pits were observed on the cambial side of the bark of Carrizo citrange rootstock (Fig. 2C) that were not correlated with any of the treatments.

Horizontal streaks of pits containing gum pockets (Fig. 2D, 2E) somewhat similar to the "wood pitting", "gum pocket" and "gummy pitting" disorders described in Argentina, South Africa and Australia, respectively (Fraser and Levitt, 1959; Fernández-Valiela *et al.,* 1965; Foguet and Oste, 1968; Schwarz and McClean, 1969; Marais *et al.,* 1996), were also observed in a few trees of several treatments.

When the experiment was terminated and the trees were pulled out, small lesions were observed in the roots of the CEVd-infected trees (Fig. 3).
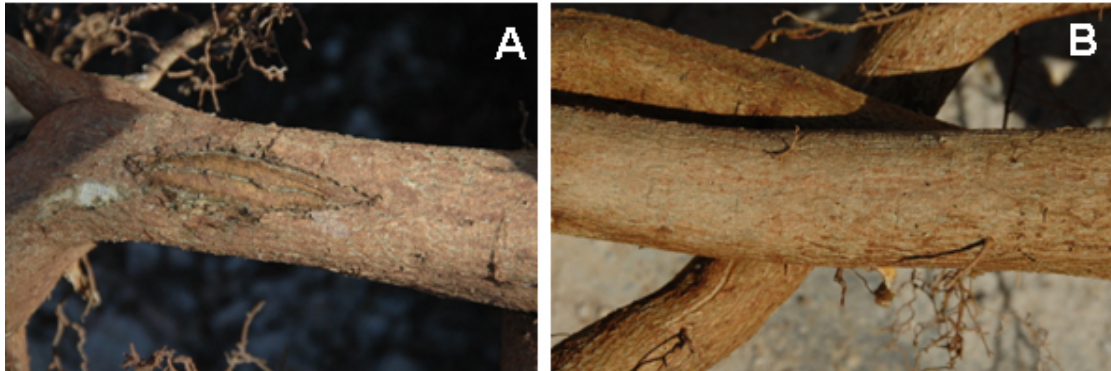
Fig. 3. (A) Lesions observed in the roots of trees infected with CEVd-117. **(B)** Roots of non infected controls.

Effect of viroid infection on tree size and fruit harvest.

Vegetative growth. As shown in Table 2A, the means of vegetative growth parameters (height, rootstock and scion circumferences and canopy volume) of trees infected with CEVd, CDVd or CBCVd were lower than those of the non-inoculated control. Only the height values were significantly different than the non-inoculated control, with CBCVd infected trees being highly significant (P=0.0002). The differences in rootstock and scion circumferences were not significant. Canopy volume was also affected as a result of viroid infection, but the effect was statistically significant (P=0.013) only in the case of CBCVd infected trees. There was no significant block and isolate effects and interactions between year*viroid and between year*isolate were not significant. When the vegetative growth parameters of each isolate were compared, the mean values of trees infected with the two CEVd isolates, the four CDVd isolates and the single CBCVd isolate were lower than that of the non-inoculated control. However, only the differences in tree height and canopy volume of CBCVd infected trees were statistically significant (P=0.0004 and P=0.031, respectively) (Table 2B).

Table 2A. Vegetative growth parameters of viroid infected Washington navel orange trees grafted on citrange Carrizo.

Treatment	Tree height (m)		Rootstock circumference (cm)		Scion circumference (cm)		Canopy volumen (m^3)	
Viroid	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	2.17	0.095	51.59	2.985	41.55	2.956	5.69	0.487
CEVd	1.89	0.076	49.72	2.516	39.46	2.244	4.44	0.349
CBLVd	2.21	0.076	54.18	2.482	42.02	2.192	5.81	0.349
HSVd	2.13	0.069	54.13	2.482	44.19	2.086	5.56	0.289
CDVd	1.90	0.064	50.13	2.370	38.69	1.964	4.70	0.259
CBCVd	1.64	0.095	46.76	3.234	40.67	3.316	3.60	0.486

P values obtained when comparing data of viroid-infected treatments with the non-inoculated controls.

Treatment	Tree height	Rootstock circumference	Scion circumference	Canopy volume
Block	0.124	0.119	0.303	.
Block*Viroid	0.357	0.327	.	0.265
UN(1,1)[1]	<.0001	–	–	<.0001
UN(2,1)	0.0002	–	–	<.0001
UN(2,2)	<.0001	–	–	<.0001
Year	<.0001	–	–	<.0001
Viroid	<.0001	0.105	0.309	0.001
Isolate	0.489	0.191	0.486	0.853
Year*Viroid	0.846	–	–	0.062
Year*Isolate)	0.061	–	–	0.829
Control vs CEVd	0.037	0.919	0.934	0.126
Control vs CBLVd	0.986	0.801	0.999	0.999
Control vs HSVd	0.991	0.798	0.838	0.998
Control vs CDVd	0.028	0.967	0.775	0.226
Control vs CBCVd	0.0002	0.453	0.999	0.013

[1]UN=Significance of the correlation between successive yearly measurements taken in the same trees.

When the experiment was terminated, the fresh weights of the roots were evaluated. As shown in Table 3A, the mean fresh weight of trees infected with CEVd or with CBCVd were smaller than those of the non-infected controls but the differences were not statistically significant. When the data from each isolate were compared, the major deviations from the non-inoculated controls were found in the case of trees infected with CBCVd, with the severe CEVd isolate (CEVd-117) or with two of the four CDVd isolates tested, but the differences were not statistically significant. Unexpectedly, the mean value of one of the HSVd isolates (HSVd-IIa-117) was higher than that of the non-inoculated controls but the difference was not statistically significant (Table 3B).

Table 2B. Vegetative growth parameters of viroid infected Washington navel orange trees grafted on citrange Carrizo.

Treatment	Tree height (m)		Rootstock circumference (cm)		Scion circumference (cm)		Canopy volumen (m³)	
Viroid isolate	Mean	*SE*	Mean	SE	Mean	*SE*	Mean	SE
Control	2.16	0.096	51.57	2.993	41.55	2.956	5.69	0.487
CEVd-117	1.84	0.096	49.91	3.236	40.92	3.316	4.15	0.487
CEVd-129	1.94	0.096	49.50	2.788	38.00	2.669	4.72	0.487
CBLVd-Ia -117	2.14	0.096	54.35	2.969	42.88	2.942	5.73	0.487
CBLVd-Ib	2.28	0.096	54.75	2.993	41.15	2.956	5.90	0.487
HSVd-IIa-117	2.14	0.096	49.17	2.993	40.55	2.956	5.28	0.487
HSVd-IIb	2.28	0.096	58.12	3.596	47.56	3.818	5.25	0.487
HSVd-IIc	1.84	0.096	54.95	2.969	44.48	2.942	6.14	0.487
CDVd-IIIa	1.86	0.096	50.87	2.993	42.25	2.956	4.68	0.487
CDVd-IIIb	1.92	0.096	54.01	3.600	36.40	3.815	4.77	0.487
CDVd-IIIc	1.90	0.104	48.30	3.233	35.22	3.303	4.49	0.534
CDVd-IIId	1.90	0.096	46.90	3.236	40.92	3.316	4.87	0.487
CBCVd	1.63	0.096	46.65	3.236	40.67	3.316	3.60	0.487

P values obtained when comparing data of viroid-infected treatments with the non-inoculated controls.

Treatment	Tree height	Rootstock circumference	Scion circumference	Canopy volumen
Block	0.108	0.114	0.303	.
UN(1,1)[1]	<.0001	–	–	<.0001
UN(2,1)	<.0001	–	–	<.0001
UN(2,2)	<.0001	–	–	<.0001
Year	<.0001	–	–	<.0001
Isolate	<.0001	0.105	0.519	0.019
Year*Isolate	0.206	–	–	0.291
Control vs CEVd-117	0.069	0.999	1.000	0.203
Control vs CEVd-129	0.358	0.998	0.965	0.722
Control vs CBLVd -Ia	1.000	0.998	1.000	1.000
Control vs CBLVd-Ib	0.962	0.995	1.000	1.000
Control vs HSVd-IIa	0.867	0.995	1.000	0.999
Control vs HSVd-IIb	0.999	0.530	0.798	0.998
Control vs HSVd-IIc	0.992	0.954	0.995	0.998
Control vs CDVd-IIIa	0.097	1.000	1.000	0.675
Control vs CDVd-IIIb	0.291	0.998	0.902	0.775
Control vs CDVd-IIIc	0.259	0.971	0.647	0.529
Control vs CDVd-IIId	0.217	0.795	1.000	0.859
Control vs CBCVd	0.0004	0.747	1.000	0.031

[1]UN=Significance of the correlation between successive yearly measurements taken in the same trees.

Table 3A. Root weight of viroid infected Washington navel orange trees grafted on citrange Carrizo.

P values obtained when comparing data of viroid-infected treatments with the non-inoculated controls

Treatment	Rootstock weight (kg)	
Viroid	Mean	SE
Control	15.67	3.640
CEVd	13.27	2.674
CBLVd	17.18	2.674
HSVd	19.53	2.261
CDVd	14.47	2.066
CBCVd	11.53	3.640

Treatment	Rootstock weight (kg)
Block	.
Block *Viroid	0.2599
Viroid	0.3624
Isolate	0.6252
Control vs CEVd	0.9625
Control vs CBLVd	0.9950
Control vs HSVd	0.7768
Control vs CDVd	0.9975
Control vs CBCVd	0.8398

Table 3B. Root weight of viroid infected Washington navel orange trees grafted on citrange Carrizo.

P values obtained when comparing data of viroid-infected treatments with the non-inoculated controls

Treatment	Rootstock weight (kg)	
Isolate	Mean	SE
Control	15.67	3.645
CEVd-117	12.37	3.645
CEVd-129	14.17	3.645
CBLVd-Ia 117	16.92	3.645
CBLVd-Ib	17.42	3.645
HSVd-IIa-117	25.84	3.645
HSVd-IIb	16.17	3.645
HSVd-IIc	16.59	3.645
CDVd-IIIa	13.67	3.645
CDVd-IIIb	15.50	3.645
CDVd-IIIc	15.30	3.992
CDVd-IIId	13.45	3.645
CBCVd	11.53	3.645

Isolate	Rootstock weight (kg)
Block	.
Isolate(Viroid)	0.5230
Control vs CEVd-117	0.9983
Control vs CEVd-129	1.0000
Control vs CBLVd-Ia	1.0000
Control vs CBLVd-Ib	1.0000
Control vs HSVd-IIa	0.3280
Control vs HSVd-IIb	1.0000
Control vs HSVd-IIc	1.0000
Control vs CDVd-IIIa	1.0000
Control vs CDVd-IIIb	1.0000
Control vs CDVd-IIIc	1.0000
Control vs CDVd-IIId	1.0000
Control vs CBCVd	0.9890

Fruit harvest.

With the exception of trees infected with CBCVd, no major differences in annual and cumulative yields were found between viroid-infected trees and the non-inoculated controls (Table 4A). No significant differences were found between the treatments and the controls.

However it should be noted that the relatively small harvest produced by CBCVd-infected trees was compensated by their small canopy (Table 4A), and resulted in an increased crop efficiency (yield/volume=20.28) that was considerably larger than that (yield/volume=15.79) of the non-inoculated controls, but the difference was not highly significant (P=0.126) (Table 4A). There was no significant block effect. The small interaction found between year*viroid was disregarded because the statistical analysis of data collected each year did not show statistical differences between the treatments and the control (data not shown). Comparison of yield data of trees infected with each isolate showed that trees infected with the severe CEVd isolate (CEVd-117) or with CBCVd produced smaller annual and cumulative yields than the non-inoculated controls, but the differences were not highly significant (P=0.651 and P=0.257, repectively)(Table 4B).

Fruit characteristics.

Only slight differences were found in color index during the fruit-ripening period, and they became essentially identical at harvesting time (Table 5A). No statistical differences were found when the values of viroid-infected trees were compared with those of the non-inoculated control (Table 5A), or when the values of the various isolates were compared (Table 5B). Slight differences were observed in some of the fruit parameters measured, which became more obvious at harvesting time (Table 6A and Table 7A). The results suggest that the fruits of the HSVd infected trees were slightly smaller (height, P=0.032; diameter, P=0.086) than those of the non-infected controls. When fruit heights and diameters of trees infected with each isolate were compared, no significant differences were found (Table 6B and Table 7B), but the results suggest that the fruits of trees infected with isolate HSVd-IIc were somewhat smaller.

The external quality parameters of fruits harvested in 2007 are shown in Table 8A. No effects on fruit shape (diameter/height), rind thickness and fruit density were found. However, as indicated above, the size of fruits from HSVd infected trees were smaller than the controls (P=0.077). When quality parameters of fruits from trees infected with each isolate were compared, no significant differences were found (Table 8B). As indicated above, the results indicate that the fruits of trees infected with isolate HSVd-IIc were somewhat smaller.

The internal quality parameters of fruits harvested in 2007 are shown in Table 9A. Fruits collected from CEVd or CDVd infected trees seem to contain less soluble solids but the differences were not highly significant (P=0.151 and P=0.165, respectively) with a minor impact on the maturity indexes. When quality parameters of fruits from trees infected with each isolate were compared, only differences in the content of soluble solids of fruits infected with one of the CDVd isolates (CDVd-IIIc) were somewhat relevant (P=0.105).(Table 9B).

Table 4A. Fruit yields of viroid infected Washington navel orange trees grafted on citrange Carrizo.

Treatment	Yield /volume[1]		Annual yield (kg)[2]		Cumulative yield (kg)[3]	
Viroid	Mean	SE	Mean	SE	Mean	SE
Control	15.79	1.533	66.42	7.062	199.25	21.371
CEVd	18.10	1.122	62.40	6.235	187.21	18.916
CBLVd	14.69	1.122	68.61	6.235	205.83	18.916
HSVd	16.66	0.946	68.31	5.934	204.92	18.022
CDVd	18.14	0.863	63.06	5.788	190.93	17.647
CBCVd	20.28	1.532	51.00	7.062	153.00	21.371

[1]Average of data collected in years 2004 and 2007.
[2]Average of data collected in 2004, 2006 and 2007 (data for 2005 was not available).
[3]Cumulative yield of data collected in 2004, 2006 and 2007 (data for 2005 was not available).

P values obtained when comparing data of viroid-infected treatments with the non-inoculated controls.

Treatment	Yield /volume	Annual yield (kg)	Cumulative yield (kg)
Bloque	0.256	0.094	0.092
Bloque*Viroid	0.252	0.036	0.057
UN(1,1)	<.0001	<.0001	–
UN(2,1)	0.448	0.002	–
UN(2,2)	<.0001	<.0001	–
UN(3,1)	–	0.142	–
UN(3,2)	–	0.011	–
UN(3,3)	–	<.0001	–
Year	<.0001	<.0001	–
Viroid	0.043	0.210	0.204
Isolate	0.083	0.964	0.947
Year*Viroid	0.029	0.178	–
Year*isolate	0.085	0.685	–
Control vs CEVd	0.533	0.964	0.961
Control vs CBLVd	0.943	0.997	0.997
Control vs HSVd	0.971	0.998	0.998
Control vs CDVd	0.449	0.978	0.989
Control vs CBCVd	0.126	0.193	0.184

Table 4B. Fruit yields of viroid infected Washington navel orange trees grafted on citrange Carrizo.

Treatment	Yield /volume		Annual yield (kg)		Cumulative yield (kg)	
Viroid isolate	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	15.79	1.524	66.42	7.208	199.25	21.864
CEVd-117	18.98	1.524	59.58	7.208	178.75	21.864
CEVd-129	17.22	1.524	65.22	7.208	195.67	21.864
CBLVd-Ia -117	14.75	1.524	66.83	7.208	200.50	21.864
CBLVd-Ib	14.63	1.524	70.39	7.208	211.17	21.864
HSVd-IIa-117	15.64	1.524	66.03	7.208	198.08	21.864
HSVd-IIb	20.65	1.524	71.33	7.208	214.00	21.864
HSVd-IIc	13.69	1.524	67.56	7.208	202.67	21.864
CDVd-IIIa	17.81	1.524	62.86	7.208	188.58	21.864
CDVd-IIIb	18.49	1.524	64.06	7.208	192.17	21.864
CDVd-IIIc	19.25	1.670	63.19	7.359	194.34	23.172
CDVd-IIId	17.07	1.524	61.56	7.208	184.67	21.864
CBCVd	20.28	1.524	51.00	7.208	153.00	21.864

1Average of data collected in years 2004 and 2007.
2Average of data collected in 2004, 2006 and 2007 (data for 2005 was not available).
3Cumulative yield of data collected in 2004, 2006 and 2007 (data for 2005 was not available).

P values obtained when comparing data of viroid-infected treatments with the non-inoculated controls.

Treatment	Yield /volume	Annual yield (kg)	Cumulative yield (kg)
Bloque	0.234	0.076	0.076
UN(1,1)[1]	<.0001	<.0001	–
UN(2,1)	0.321	0.001	–
UN(2,2)	<.0001	<.0001	–
UN(3,1)	–	0.007	–
UN(3,2)	–	0.001	–
UN(3,3)	–	<.0001	–
Año	<.0001	<.0001	–
raza	0.025	0.563	0.568
Año*raza	0.021	0.405	–
Control vs CEVd-117	0.651	0.977	0.978
Control vs CEVd-129	0.997	1.000	1.000
Control vs CBLVd-Ia	0.999	1.000	1.000
Control vs CBLVd-Ib	0.999	0.999	0.999
Control vs HSVd-IIa	1.000	1.000	1.000
Control vs HSVd-IIb	0.184	0.998	0.998
Control vs HSVd-IIc	0.952	1.000	1.000
Control vs CDVd-IIIa	0.962	0.999	0.999
Control vs CDVd-IIIb	0.815	1.000	1.000
Control vs CDVd-IIIc	0.615	1.000	1.000
Control vs CDVd-IIId	0.999	0.998	0.998
Control vs CBCVd	0.257	0.322	0,324

Table 5A. Color evolution of fruits from viroid infected Washington navel sweet orange trees grafted on Carrizo citrange.

Treatment	Color 1[1]		Color 2		Color 3		Color 4		Color 5		Color 6	
viroid	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	-11.03	0.719	0.81	1.165	7.99	0.676	10.02	0.406	12.04	0.317	14.57	0.504
CEVd	-10.88	0.560	0.31	1.016	7.30	0.602	10.36	0.316	11.95	0.241	14.49	0.367
CBLVd	-10.61	0.560	0.22	1.016	7.76	0.602	9.16	0.316	12.19	0.241	14.32	0.367
HSVd	-10.84	0.496	-0.17	0.962	7.38	0.574	9.77	0.280	12.04	0.209	14.47	0.308
CDVd	-11.39	0.467	0.38	0.938	7.12	0.563	9.70	0.264	11.94	0.195	14.71	0.28
CBCVd	-12.67	0.719	1.74	1.165	5.93	0.676	9.25	0.404	12.25	0.316	15.08	0.504

[1] Color was evaluated with a random sample of ten fruits per tree. Data was collected monthly from October until harvesting time.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Color 1	Color 2	Color 3	Color 4	Color 5	Color 6
Block	0.164	0.072	0.129	0.110	0.134	0.242
Block*Viroid	0.328	0.428	0.079	.	.	.
Block* Isolate	0.007	2.000	0.013	0.436	0.023	0.017
Viroid	0.224	0.526	0.232	0.028	0.894	0.832
Isolate	0.915	0.892	0.340	0.924	0.023	0.261
Control vs CEVd	0.999	0.970	0.816	0.854	0.999	0.999
Control vs CBLVd	0.968	0.944	0.998	0.166	0.984	0.983
Control vs HSVd	0.999	0.684	0.865	0.940	1.000	0.999
Control vs CDVd	0.978	0.979	0.628	0.840	0.997	0.999
Control vs CBCVd	0.256	0.838	0.069	0.356	0.872	0.872

Table 5B. Color evolution of fruits from viroid infected Washington navel sweet orange trees grafted on Carrizo citrange.

Treatment	Color 1		Color 2		Color 3		Color 4		Color 5		Color 6	
Isolate	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	-11.03	0.713	0.81	1.162	7.99	0.629	10.03	0.405	12.04	0.317	14.57	0.504
CEVd-117	-11.02	0.713	0.19	1.162	7.22	0.629	10.27	0.405	12.01	0.317	13.84	0.504
CEVd-129	-10.74	0.713	0.43	1.162	7.38	0.629	10.44	0.405	11.89	0.317	15.15	0.504
CBLVd-Ia-117	-10.48	0.713	0.47	1.162	7.97	0.629	8.99	0.405	12.01	0.317	14.34	0.504
CBLVd-Ib	-10.74	0.713	-0.03	1.162	7.54	0.629	9.33	0.405	12.36	0.317	14.30	0.504
HSVd-IIa -117	-11.28	0.713	-0.94	1.162	6.79	0.629	9.57	0.405	12.32	0.317	14.57	0.504
HSVd-IIb	-10.39	0.713	0.44	1.162	8.23	0.629	9.97	0.405	11.91	0.317	15.06	0.504
HSVd-IIc	-10.83	0.713	-0.01	1.162	7.12	0.629	9.77	0.405	11.90	0.317	13.76	0.504
CDVd -IIIa	-11.56	0.713	-0.28	1.162	6.95	0.629	9.96	0.405	12.06	0.317	14.98	0.504
CDVd -IIIb	-11.87	0.713	0.30	1.162	6.90	0.629	9.62	0.405	11.80	0.317	14.13	0.504
CDVd -IIIc	-10.71	0.777	0.84	1.223	7.64	0.679	9.80	0.439	12.79	0.344	14.78	0.504
CDVd -IIId	-11.37	0.713	0.69	1.162	6.89	0.629	9.41	0.405	11.11	0.317	14.93	0.504
CBCVd	-12.67	0.713	1.74	1.162	5.93	0.629	9.25	0.405	12.25	0.317	15.08	0.504

[1] Color was evaluated in a random sample of ten fruits per tree. Data was collected monthly from October until harvesting time.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Color 1	Color 2	Color 3	Color 4	Color 5	Color 6
Block	0.158	0.071	0.106	0.110	0.134	0.242
Block*Isolate	0.000	<.0001	<.0001	0.436	0.023	0.017
Isolate	0.568	0.831	0.274	0.221	0.101	0.515
Control vs CEVd-117	1.000	1.000	0.951	1.000	1.000	0.922
Control vs CEVd-129	1.000	1.000	0.992	0.985	1.000	0.985
Control vs CBLVd -Ia	0.999	1.000	1.000	0.302	1.000	1.000
Control vs CBLVd-Ib	1.000	0.995	1.000	0.755	0.990	1.000
Control vs HSVd-IIa	1.000	0.646	0.633	0.980	0.997	0.996
Control vs HSVd-IIb	0.997	1.000	1.000	1.000	1.000	0.872
Control vs HSVd-IIc	1.000	0.996	0.905	1.000	1.000	0.999
Control vs CDVd-IIIa	1.000	0.965	0.784	1.000	1.000	0.998
Control vs CDVd-IIIb	0.977	1.000	0.738	0.991	1.000	1.000
Control vs CDVd-IIIc	1.000	1.000	1.000	1.000	0.469	1.000
Control vs CDVd-IIId	1.000	1.000	0.727	0.860	0.194	1.000
Control vs CBCVd	0.480	0.989	0.086	0.643	1.000	0.995

Table 6A. Growth (height) of fruits from viroid infected Washington navel sweet orange trees grafted on Carrizo citrange.

Treatment	Height 1 (mm)		Height 2 (mm)		Height 3 (mm)		Height 4 (mm)		Height 5 (mm)		Height 6 (mm)	
Viroid	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	71.86	0.853	75.42	1.164	77.25	1.17	79.05	1.254	77.71	1.178	84.49	1.237
CEVd	71.62	0.608	75.28	0.967	77.58	0.95	76.46	1.019	78.32	0.914	82.47	0.945
CBLVd	72.06	0.608	75.09	0.967	76.57	0.95	76.24	1.019	77.79	0.914	82.33	0.945
HSVd	71.47	0.501	74.78	0.892	76.95	0.86	76.87	0.927	78.21	0.806	80.97	0.825
CDVd	71.87	0.449	75.66	0.859	77.04	0.82	76.80	0.886	78.13	0.758	83.14	0.769
CBCVd	72.38	0.853	76.25	1.164	79.90	1.17	79.25	1.256	77.30	1.178	84.56	1.237

[1] Fruit height was measured in a random sample of ten fruits per tree. Data was collected monthly from October until harvesting time.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls .

Treatment	Height 1	Height 2	Height 3	Height 4	Height 5	Height 6
Block	.	0.155	0.249	0.096	.	0.131
Block*Viroid	0.461	0.072	0.123	0.399	0.101	.
Block* Isolate	0.006	0.087	0.014	0.011	0.005	0.0003
Viroid	0.944	0.899	0.313	0.121	0.982	0.033
Isolate	0.925	0.946	0.915	0.999	0.425	0.696
Control vs CEVd	0.999	1.000	0.999	0.163	0.989	0.388
Control vs CBLVd	0.999	0.999	0.979	0.116	1.000	0.330
Control vs HSVd	0.988	0.977	0.999	0.245	0.994	0.032
Control vs CDVd	1.000	0.999	0.999	0.204	0.997	0.653
Control vs CBCVd	0.983	0.962	0.303	0.999	0.999	1.000

Table 7A. Growth (diameter) of fruits from viroid infected Washington navel sweet orange trees grafted on Carrizo citrange.

Treatment viroid	Diameter 1 (mm)		Diameter 2 (mm)		Diameter 3 (mm)		Diameter 4 (mm)		Diameter 5 (mm)		Diameter 6 (mm)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	72.49	0.731	75.96	0.808	78.24	0.952	77.99	0.978	78.35	0.999	83.85	1.493
CEVd	71.27	0.521	75.30	0.656	77.81	0.782	77.03	0.781	78.53	0.835	81.88	1.100
CBLVd	71.18	0.429	75.91	0.656	77.37	0.782	77.34	0.780	78.34	0.835	80.12	1.100
HSVd	71.44	0.521	75.32	0.597	77.33	0.716	77.25	0.703	78.29	0.773	80.1	0.933
CDVd	71.91	0.384	76.23	0.571	77.72	0.688	77.69	0.668	78.33	0.746	81.48	0.853
CBCVd	71.56	0.731	76.31	0.808	79.54	0.952	79.34	0.978	78.99	0.999	83.71	1.492

[1]Fruit diameter was measured in a random sample of ten fruits per tree. Data was collected monthly from October until harvesting time.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Diameter 1 (mm)	Diameter 2 (mm)	Diameter 3 (mm)	Diameter 4 (mm)	Diameter 5 (mm)	Diameter 6 (mm)
Block	0.398	0.170	0.139	0.107	0.264	0.198
Block*Viroid	.	0.105	0.140	0.400	0.042	.
Block* Isolate	0.0003	0.074	0.019	0.029	0.042	0.0002
Viroid	0.596	0.716	0.393	0.344	0.994	0.095
Isolate	0.936	0.985	0.609	0.973	0.206	0.967
Control vs CEVd	0.446	0.908	0.990	0.757	1.000	0.609
Control vs	0.569	1.000	0.851	0.927	1.000	0.115
Control vs HSVd	0.333	0.904	0.807	0.869	1.000	0.086
Control vs CDVd	0.89	0.997	0.971	0.996	1.000	0.376
Control vs	0.769	0.996	0.672	0.615	0.981	1.000

Table 6B. Growth (height) of fruits from viroid infected Washington navel sweet orange trees grafted on Carrizo citrange.

Treatment	Height 1 (mm)		Height 2 (mm)		Height 3 (mm)		Height 4 (mm)		Height 5 (mm)		Height 6 (mm)	
Isolate	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	71.86	0.853	75.42	1.166	77.25	1.159	79.05	1.256	77.71	1.181	84.49	1.238
CEVd-117	72.06	0.853	75.06	1.166	77.44	1.159	76.44	1.256	76.96	1.181	82.35	1.238
CEVd-129	71.17	0.853	75.50	1.166	77.72	1.159	76.49	1.256	79.68	1.181	82.59	1.238
CBLVd-Ia-117	72.53	0.853	75.68	1.166	77.23	1.159	76.43	1.256	77.55	1.181	83.15	1.238
CBLVd-Ib	71.58	0.853	74.50	1.166	75.91	1.159	76.05	1.256	78.02	1.181	81.51	1.238
HSVd-IIa -117	71.72	0.853	74.88	1.166	77.31	1.159	77.20	1.256	79.28	1.181	81.13	1.238
HSVd-IIb	71.54	0.853	74.95	1.166	76.43	1.159	76.77	1.256	76.88	1.181	81.31	1.238
HSVd-IIc	71.16	0.853	74.50	1.166	77.11	1.159	76.64	1.256	78.47	1.181	80.47	1.238
CDVd -IIIa	71.12	0.853	75.02	1.166	76.24	1.159	76.83	1.256	78.22	1.181	82.47	1.238
CDVd -IIIb	72.01	0.853	76.26	1.166	76.81	1.159	77.11	1.256	77.89	1.181	82.37	1.238
CDVd -IIIc	72.22	0.934	75.48	1.264	77.66	1.269	76.55	1.348	79.07	1.294	85.05	1.344
CDVd -IIId	72.13	0.853	75.95	1.166	77.46	1.159	76.71	1.256	77.42	1.181	82.67	1.238
CBCVd	72.38	0.853	76.25	1.166	79.90	1.159	79.24	1.256	77.29	1.181	84.56	1.238

[1]Fruit heights was measured in a random sample of ten fruits per tree. Data was collected monthly from October until harvesting time.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Height 1 (mm)	Height 2 (mm)	Height 3 (mm)	Height 4 (mm)	Height 5 (mm)	Height 6 (mm)
Block	.	0.120	0.226	0.093	.	0.131
Block*Isolate	0.0002	0.005	0.000	0.002	<.0001	0.0003
Isolate	0.987	0.984	0.703	0.593	0.875	0.166
Control vs CEVd-117	1.000	1.000	1.000	0.474	1.000	0.772
Control vs CEVd-129	0.999	1.000	1.000	0.497	0.870	0.865
Control vs CBLVd -Ia	0.999	1.000	1.000	0.470	1.000	0.984
Control vs CBLVd-Ib	1.000	0.999	0.983	0.313	1.000	0.393
Control vs HSVd-IIa	1.000	1.000	1.000	0.839	0.965	0.261
Control vs HSVd-IIb	1.000	1.000	1.000	0.639	1.000	0.319
Control vs HSVd-IIc	0.999	0.999	1.000	0.572	1.000	0.112
Control vs CDVd-IIIa	0.999	1.000	0.998	0.667	1.000	0.822
Control vs CDVd-IIIb	1.000	1.000	1.000	0.802	1.000	0.778
Control vs CDVd-IIIc	1.000	1.000	1.000	0.593	0.992	1.000
Control vs CDVd-IIId	1.000	1.000	1.000	0.609	1.000	0.892
Control vs CBCVd	1.000	1.000	0.523	1.000	1.000	1.000

Table 7B. Growth (diameter) of fruits from viroid infected Washington navel sweet orange trees grafted on Carrizo citrange

Treatment	Diameter 1 (mm)		Diameter 2 (mm)		Diameter 3 (mm)		Diameter 4 (mm)		Diameter 5 (mm)		Diameter 6 (mm)	
Isolate	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	72.49	0.731	75.96	0.813	78.25	0.946	78.25	0.946	78.25	0.946	83.85	1.493
CEVd-117	71.12	0.731	75.32	0.813	77.29	0.946	77.29	0.946	77.29	0.946	81.8	1.493
CEVd-129	71.43	0.731	75.28	0.813	78.33	0.946	78.33	0.946	78.33	0.946	81.99	1.493
CBLVd-Ia-117	71.53	0.731	76.21	0.813	77.83	0.946	77.83	0.946	77.83	0.946	80.57	1.493
CBLVd-Ib	71.35	0.731	75.61	0.813	76.92	0.946	76.92	0.946	76.92	0.946	79.66	1.493
HSVd-IIa -117	71.45	0.731	75.53	0.813	77.3	0.946	77.3	0.946	77.3	0.946	79.47	1.493
HSVd-IIb	70.91	0.731	74.93	0.813	76.92	0.946	76.92	0.946	76.92	0.946	81.07	1.493
HSVd-IIc	71.18	0.731	75.5	0.813	77.78	0.946	77.78	0.946	77.78	0.946	79.76	1.493
CDVd -IIIa	72.07	0.731	76.37	0.813	77.46	0.946	77.46	0.946	77.46	0.946	80.72	1.493
CDVd -IIIb	71.1	0.731	75.84	0.813	76.92	0.946	76.92	0.946	76.92	0.946	81.42	1.493
CDVd -IIIc	72.54	0.805	76.35	0.883	78.94	1.025	78.94	1.025	78.94	1.025	82.65	1.633
CDVd -IIId	71.95	0.731	76.38	0.813	77.56	0.946	77.56	0.946	77.56	0.946	81.11	1.493
CBCVd	71.56	0.731	76.31	0.813	79.54	0.946	79.54	0.946	79.54	0.946	83.71	1.493

[1]Fruit diameters were measured in a random sample of ten fruits per tree. Data was collected monthly from October until harvesting time.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Diameter 1 (mm)	Diameter 2 (mm)	Diameter 3 (mm)	Diameter 4 (mm)	Diameter 5 (mm)	Diameter 6 (mm)
Block	0.397	0.130	0.119	0.102	0.148	0.198
Block*Isolate	0.0003	0.007	0.001	0.007	0.001	0.0002
Isolate	0.912	0.948	0.579	0.797	0.807	0.481
Control vs CEVd-117	0.777	0.999	0.990	0.999	1.000	0.944
Control vs CEVd-129	0.937	0.998	1.000	0.939	0.984	0.968
Control vs CBLVd -Ia	0.968	1.000	1.000	1.000	1.000	0.556
Control vs CBLVd-Ib	0.904	1.000	0.909	0.986	1.000	0.276
Control vs HSVd-IIa	0.946	1.000	0.990	1.000	0.988	0.230
Control vs HSVd-IIb	0.626	0.954	0.910	0.956	0.905	0.745
Control vs HSVd-IIc	0.815	1.000	1.000	1.000	1.000	0.299
Control vs CDVd-IIIa	1.000	1.000	0.998	1.000	1.000	0.615
Control vs CDVd-IIIb	0.760	1.000	0.910	0.999	1.000	0.858
Control vs CDVd-IIIc	1.000	1.000	1.000	1.000	1.000	1.000
Control vs CDVd-IIId	1.000	1.000	1.000	1.000	1.000	0.757
Control vs CBCVd	0.975	1.000	0.917	0.897	1.000	1.000

Table 8A. External quality parameters of fruits from viroid infected Washington navel sweet orange trees grafted on citrange Carrizo.

Treatment	Diameter/Height[1]		Rind thickness (mm)[1]		Fruit weight (g)[2]		Fruit size (ml)[2]		Fruit density (g/ml)	
Viroid	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	1.01	0.009	5.10	0.120	2933.3	131.14	3278.7	147.88	0.90	0.006
CEVd	1.01	0.007	5.08	0.091	2747.5	98.21	3098.7	111.73	0.89	0.005
CBLVd	1.03	0.007	5.16	0.091	2667.5	98.21	3008.5	111.73	0.89	0.006
HSVd	1.01	0.005	4.99	0.079	2623.3	84.43	2910.7	96.72	0.90	0.004
CDVd	1.02	0.005	4.93	0.074	2825.6	77.93	3129.4	89.68	0.90	0.004
CBCVd	1.01	0.009	5.09	0.120	3041.3	131.14	3378.5	147.88	0.90	0.006

[1]Diameter, height and rind thickness are the average measurements of the ten fruits sampled from each tree.
[2]Fruit weight and size were measured as the overall weight and volume of samples of ten fruits collected from each tree.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Diameter/Height	Rind thickness (mm)	Fruit weight (g)	Fruit size (ml)	Fruit density (g/ml)
Block	.	0.460	0.159	0.145	0.250
Block*Viroid	.	0.160	.	.	.
Block* Isolate	0.003	0.115	-	-	-
Viroid	0.154	0.432	0.033	0.041	0.014
Isolate	0.811	0.032	0.829	0.775	0.189
Control vs CEVd	1.000	1.000	0.530	0.643	0.785
Control vs CBLVd	0.188	0.985	0.234	0.306	0.554
Control vs HSVd	0.926	0.897	0.104	0.077	0.694
Control vs CDVd	0.377	0.574	0.845	0.719	0.623
Control vs CBCVd	1.000	1.000	0.927	0.964	0.990

Table 8B. External quality parameters of fruits from viroid infected Washington navel sweet orange trees grafted on citrange Carrizo.

Treatment Isolate	Diameter /Height Mean	SE	Rind thickness (mm) Mean	SE	Fruit weight (g) Mean	SE	Fruit size (ml) Mean	SE	Density (g/ml) Mean	SE
Control	1.01	0.009	5.10	0.120	2933.3	131.14	3278.7	147.88	0.90	0.006
CEVd-117	1.01	0.009	5.02	0.120	2742.0	131.14	3116.7	147.88	0.88	0.006
CEVd-129	1.01	0.009	5.13	0.120	2753.0	131.14	3080.7	147.88	0.90	0.006
CBLVd-1a-117	1.03	0.009	5.13	0.120	2753.7	131.14	3107.7	147.88	0.89	0.006
CBLVd-Ib	1.02	0.009	5.19	0.120	2581.3	131.14	2909.3	147.88	0.89	0.006
HSVd-IIa -117	1.02	0.009	4.84	0.120	2657.0	131.14	2931.0	147.88	0.91	0.006
HSVd-IIb	1.00	0.009	5.35	0.120	2645.0	131.14	2981.3	147.88	0.89	0.006
HSVd-IIc	1.01	0.009	4.79	0.120	2568.0	131.14	2819.7	147.88	0.91	0.006
CDVd -IIIa	1.02	0.009	4.87	0.120	2789.2	131.14	3089.0	147.88	0.90	0.006
CDVd -IIIb	1.01	0.009	4.96	0.120	2790.3	131.14	3097.2	147.88	0.90	0.006
CDVd -IIIc	1.03	0.010	4.99	0.132	2990.8	143.02	3310.6	161.00	0.90	0.007
CDVd -IIId	1.02	0.009	4.89	0.120	2732.0	131.14	3020.7	147.88	0.91	0.006
CBCVd	1.01	0.009	5.09	0.120	3041.3	131.14	3378.5	147.88	0.90	0.006

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Diameter/Height	Rind thickness (mm)	Fruit weight (g)	Fruit size (ml)	Density (g/ml)
Block	.	0.420	0.159	0.145	0.250
Block*Isolate	0.003	0.020	.	.	.
Isolate	0.467	0.072	0.206	0.210	0.024
Control vs CEVd-117	1.000	1.000	0.909	0.984	0.643
Control vs CEVd-129	1.000	1.000	0.935	0.940	1.000
Control vs CBLVd -1a	0.379	1.000	0.937	0.978	0.893
Control vs CBLVd-Ib	0.743	1.000	0.300	0.368	0.964
Control vs HSVd-IIa	0.805	0.649	0.585	0.440	0.780
Control vs HSVd-IIb	1.000	0.636	0.535	0.627	0.993
Control vs HSVd-IIc	1.000	0.421	0.261	0.155	0.377
Control vs CDVd-IIIa	0.893	0.767	0.986	0.955	0.964
Control vs CDVd-IIIb	0.999	0.989	0.986	0.966	1.000
Control vs CDVd-IIIc	0.511	0.999	1.000	1.000	0.988
Control vs CDVd-IIId	0.867	0.839	0.881	0.775	0.893
Control vs CBCVd	1.000	1.000	0.999	1.000	1.000

Table 9A. Internal quality parameters of fruits from viroid infected Washington navel Orange grafted on citrange Carrizo.

Treatment viroid	Juice volume (ml)[1]		Juice density (g/ml)		Soluble solids (%)		Total acidity (%)		Maturity index	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	1156.7	53.10	1.05	0.004	14.00	0.215	1.48	0.109	9.64	0.749
CEVd	1079.7	41.27	1.05	0.003	13.50	0.160	1.30	0.082	10.77	0.561
CBLVd	1079.7	41.27	1.06	0.003	13.89	0.160	1.37	0.082	10.39	0.561
HSVd	1041.1	36.49	1.05	0.003	13.89	0.136	1.42	0.071	9.94	0.483
CDVd	1109.3	34.34	1.05	0.003	13.55	0.125	1.30	0.065	10.90	0.446
CBCVd	1185.8	53.10	1.05	0.004	13.60	0.215	1.29	0.109	10.83	0.749

[1]Juice extracted from samples of ten randomly collected fruits.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Juice volume	Juice density	Soluble solid	Total acidity	Maturity index
Block	0.191	0.410	0.181	0.1542	0.158
Block*Viroid	0.274	.	.	.	.
Viroid	0.185	0.698	0.072	0.4011	0.401
Isolate	0.302	0.666	0.334	0.9226	0.923
Control vs CEVd	0.539	0.475	0.151	0.4715	0.472
Control vs CBLVd	0.539	0.909	0.982	0.7862	0.786
Control vs HSVd	0.177	0.976	0.979	0.9907	0.991
Control vs CDVd	0.833	0.638	0.165	0.3100	0.310
Control vs CBCVd	0.987	0.596	0.429	0.5555	0.556

Table 9B. Internal quality parameters of fruits from viroid infected Washington navel Orange grafted on citrange Carrizo.

Treatment Isolate	Juice volume (ml) Mean	SE	Density juice (g/ml) Mean	SE	Soluble solids (%) Mean	SE	Total acidity (%) Mean	SE	Maturity index Mean	SE
Control	1156.7	53.32	1.05	0.003	14.0	0.215	1.483	0.109	9.64	0.749
CEVd-117	1088.3	53.32	1.05	0.003	13.5	0.215	1.387	0.109	10.42	0.749
CEVd-129	1070.0	53.32	1.05	0.003	13.5	0.215	1.222	0.109	11.12	0.749
CBLVd-Ia-117	1123.3	53.32	1.06	0.003	13.9	0.215	1.350	0.109	10.35	0.749
CBLVd-Ib	1035.0	53.32	1.06	0.003	13.9	0.215	1.383	0.109	10.42	0.749
HSVd-IIa -117	981.7	53.32	1.06	0.003	13.9	0.215	1.465	0.109	9.58	0.749
HSVd-IIb	1090.0	53.32	1.05	0.003	13.7	0.215	1.382	0.109	10.03	0.749
HSVd-IIc	1051.7	53.32	1.06	0.003	14.1	0.215	1.418	0.109	10.20	0.749
CDVd -IIIa	1103.3	53.32	1.05	0.003	13.6	0.215	1.233	0.109	11.42	0.749
CDVd -IIIb	1080.0	53.32	1.05	0.003	13.5	0.215	1.280	0.109	11.04	0.749
CDVd -IIIc	1194.9	58.06	1.05	0.003	13.2	0.235	1.235	0.119	10.94	0.817
CDVd -IIId	1058.3	53.32	1.05	0.003	14.0	0.215	1.445	0.109	10.20	0.749
CBCVd	1185.8	53.32	1.05	0.003	13.6	0.215	1.285	0.109	10.83	0.749

[1] Juice extracted from samples of ten randomly collected fruits.

P values obtained when comparing data of viroid infected treatments with the non-inoculated controls.

Treatment	Juice volume	Density juice	Soluble solid	Total acidity	Maturity index
Block	0.145	0.41	0.181	0.154	0.158
Isolate	0.184	0.781	0.135	0.66	0.797
Control vs CEVd-117	0.955	0.89	0.483	0.998	0.99
Control vs CEVd-129	0.835	0.89	0.479	0.425	0.655
Control vs CBLVd -Ia	1	0.89	1	0.969	0.995
Control vs CBLVd-Ib	0.477	1	1	0.997	0.99
Control vs HSVd-IIa	0.116	0.89	1	1	1
Control vs HSVd-IIb	0.962	1	0.969	0.996	1
Control vs HSVd-IIc	0.652	1	1	1	1
Control vs CDVd-IIIa	0.993	0.89	0.649	0.48	0.439
Control vs CDVd-IIIb	0.911	1	0.366	0.718	0.717
Control vs CDVd-IIIc	1	0.911	0.105	0.55	0.828
Control vs CDVd-IIId	0.723	0.89	1	1	1
Control vs CBCVd	1	0.89	0.734	0.743	0.859

Discussion

The routine use of Etrog citron as the exocortis indicator for biological indexing purposes revealed a range of symptoms that were erroneously considered as evidences for the existence of different CEVd stains until viroids other than CEVd were identified and shown to induce specific symptoms in this indicator (Duran-Vila *et al.,* 1986; 1988). Since naturally occurring field isolates contain more than a single viroid, the effect of each viroid on field grown trees was not established until 2004 when the results of a long-term field assay performed with clementine trees grafted on the trifoliate orange rootstock became available (Vernière *et al.,* 2004). Additional long-term field assays also revealed that viroid-induced effects might be attenuated or enhanced when trees were exposed to mixed viroid infections (Vernière *et al.,* 2006).

Field observations indicated that trees grafted on Troyer and Carrizo citranges were also viroid sensitive and developed mild exocortis symptoms, but only the effects of uncharacterized field sources or isolates containing several viroids have been reported (Polizzi *et al.,* 1991; Bani Hashemian *et al.,* 2009). The present study reports the results of an attempt to establish the effect of single viroid sources on the performance of Washington navel sweet orange trees grafted on Carrizo citrange.

Unexpectedly, no bark scaling symptoms characteristic of the exocortis disease were observed as result viroid infection. However CEVd infected trees presented small lesions in the roots, a mild form of the symptoms recently described in trees grafted on Carrizo citrange that were infected with a field isolate containing CEVd, HSVd, CBLVd and CDVd (Bani Hashemian *et al.,* 2009). Several trees presented bark-cracking symptoms but they were only consistently observed in trees infected with CBCVd or with the mild CEVd isolate (CEVd-129) as well as in the non-infected controls. In most instances the bark cracking symptoms were mild and extended above the bud-union suggesting that they might be the result of sunburn and not associated with viroid infection. However in the case of CBCVd infected trees, bark cracking was intense and mainly restricted to the rootstock suggesting that it was a viroid induced effect as demonstrated earlier in trees grafted on trifoliate orange (Vernière *et al.,* 2004). As also reported earlier (Vernière *et al.,* 2004), the presence of pegs and gummy pits was not associated with viroid infection.

Infection with CEVd or CDVd also caused a slight reduction of tree growth and yield that was not as significant as reported in the case of trees grafted on trifoliate orange (Vernière *et al.,* 2004). Unexpectedly, CBCVd that did not affect the growth of trees grafted on trifoliate orange, showed the most significant reduction of tree size (height and canopy volume). As

reported by Bani Hashemian *et al.* (2009) reduction of tree size was associated with a small root system.

CBCVd infected trees, in spite of producing smaller yields than the non-infected controls, resulted in large yield efficiency (yield/canopy volume). Unfortunately the P values were not small enough to infer if the differences were statistically significant. Further assays with larger numbers of trees will be necessary to confirm the sensitivity of Carrizo citrange to CBCVd infection and to postulate its usefulness to control tree size in high-density plantations.

With the exception of fruits harvested from HSVd infected trees that presented a smaller caliber, viroid infection did not affect any of the fruit quality parameters evaluated.

In summary, the results of this study indicate that under the growing conditions in which the assay was performed, viroid infection has little or no effect on the performance of Washington navel trees grafted on Carrizo citrange. However, these results must be taken with caution because field isolates usually contain several viroids and the effect of mixed infections has been shown to affect the performance of the trees as has been recently shown in a field assay conducted with 'Nules' clementine and 'Navelina' sweet orange tress also grafted on Carrizo citrange (Bani Hashemian *et al.,* 2009). If the sensitivity of Carrizo citrange to CBCVd is confirmed, this viroid may be the best candidate to control the size of trees grafted on Carrizo citrange in high density planting.

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Conclusiones

Conclusiones

Los trabajos desarrollados y que se hallan incluidos en esta memoria de tesis doctoral permiten llegar a las siguientes conclusiones:

1. Se ha desarrollado un protocolo de hibridación en formato *northern* con sondas marcadas con digoxigenina específicas para los distintos viroides descritos en cítricos, que permite la detección de todos los viroides sin necesidad de recurrir a la inoculación de cidro Etrog como huésped bioamplificador.

2. La sensibilidad de la técnica se ha evaluado utilizando muestras de varias especies y cultivares de cítricos recolectadas en distintas épocas del año. La técnica es extremadamente sensible, de manipulación sencilla y permite acortar el tiempo necesario para detectar viroides de forma fiable y segura.

3. La disponibilidad de dicha técnica ha permitido realizar prospecciones en tres países citrícolas (Colombia, Perú y Brasil).

4. En limas Tahití de Colombia se han identificado infecciones múltiples con HSVd y CDVd o con CEVd, HSVd y CDVd. Se han secuenciado las variantes de dichos viroides y se ha determinado su relación filogenética con las correspondientes secuencias de referencia.

5. La prospección realizada en Colombia ha permitido identificar un aislado de CEVd que aparentemente no induce síntomas en cidro Etrog. La secuencia representativa de dicho aislado presenta una identidad de secuencia del 98,9% con la secuencia de referencia que induce síntomas muy agresivos en cidro Etrog. Ambas variantes se diferencian en U186A (dominio TR), A235U (dominio V) y AU313-314GA (dominio P). Dada la implicación del dominio P en la patogénesis del CEVd, se han obtenido mutantes en las posiciones 313-314 para demostrar si los cambios identificados son efectivamente los responsables de que los cidros infectados no manifiesten síntomas.

6. El análisis de clones de lima Tahití procedentes de Perú y Brasil que manifiestan el síndrome del "quebra-galho", ha mostrado que se encuentran infectados con CEVd, HSVd y CDVd (Perú) o con CEVd y CDVd (Brasil). Los resultados preliminares de un estudio realizado en condiciones de campo para determinar si estos viroides son los responsables del síndrome del "quebra-galho" muestran que el CEVd causa agrietado de la corteza en lima Tahití y probablemente sea el responsable "quebra-galho".

7. La caracterización molecular de 33 aislados que contenían CDVd ha permitido observar que contienen poblaciones más o menos homogéneas de variantes de secuencia. Para cada aislado se ha identificado y secuenciado la/s variante/s más representativa. Con dichas variantes se ha construido un árbol filogenético y se ha establecido su relación con las secuencias de referencia (CVd-IIIa, CVd-IIIb y CVd-IIIc).

8. En base a la distribución de las distintas variantes dentro del árbol filogenético, se seleccionaron 5 secuencias representativas de los distintos grupos filogenéticos para su caracterización biológica en cidro Etrog. La expresión de síntomas y los datos de crecimiento han demostrado que dos variantes son virulentas, dos presentan una virulencia moderada y una es avirulenta. Los cambios encontrados en estas variante se han correlacionado con la modulación de los síntomas.

9. En un ensayo establecido hace quince años con naranjos Washington navel injertados en citrange Carrizo en el que se inocularon doce aislados de cinco viroides se encontraron diferencias en los parámetros cuantitativos analizados, pero no fueron estadísticamente significativas.

Anejos