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Homology of Hop Stunt Viroid with Citrus Cachexia Viroid

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ABSTRACT. Viroid nucleic acids of field trees infected by two California isolates of cachexia, Ca 908 and Ca 904 (U.C.R. virus collection), and from two Sicilian commercial citrus orchards infected by different viroids were obtained by phenol extraction and CF-11 cellulose chromatography. Extracts electrophoresed in bidirectional polyacrylamide gels under denaturing condition (dPAGE) showed positive results in all seasons except winter, with different electrophoretic patterns. Electrophoresed extracts, after blotting on nylon membranes, were hybridized with citrus exocortis viroid (CEVd)- or hop stunt viroid (HSVd)-specific cDNA probes. Of all tested isolates cachexia viroid and other viroids belonging to group II found in Sicilian commercial citrus did not react with CEVd probe, but showed positive reaction with HSVd probe. These results clearly showed homology between two isolates of cachexia, other viroids of group II and hop stunt viroid.

Index words. citrus viroid, indexing, hybridization assays.

Cachexia is a term introduced by Childs (3) to describe a disease of Orlando tangelo. Until a few years ago the disease was considered to be caused by a citrus virus or virus-like agent although the causal agent was not described. The viroid hypothesis was introduced by Roistacher, *et al.* (12) because of the similarity in transmission properties between the cachexia agent and the citrus exocortis viroid (CEVd), the lack of evidence for vector transmission, the ineffectiveness of thermotherapy (2) and the elimination of the agent by shoot tip grafting (13).

Cachexia is widespread in many citrus areas of the world. In Sicily the disease is a serious problem for Mapo tangelo and different selections of Satsuma. The lengthy and laborious bioassay requires an incubation period of 2-7 yr in Orlando tangelo and 6-18 months in Parson's Special mandarin (11).

Recently Semancik *et al.* (14) showed that a viroid is the causal agent of cachexia disease and suggested the name of "citrus cachexia viroid" (CCaV).

The purpose of the present study was to determine if this new viroid is homologous to known viroids, and to find suitable tissues and quick proce-

dures for reliably detecting CCaVd in samples collected in field.

MATERIALS AND METHODS

Viroids. Two different isolates of citrus cachexia viroid, reported as Ca 904 and Ca 908 kindly supplied by C. N. Roistacher, University of California, Riverside, U.S.A. were used in this study. They have been compared with different viroids discovered many years ago on commercial trees of Femminello Fior d'arancio (F.F.D.A.) and Continella Femminello (F.C.) lemons that induced gum formation in Parson's Special mandarin (4).

Plant materials. Test trees were commercial F.F.D.A. and F.C. lemons about 10 yr old. Other plant material used was Parson's Special mandarin inoculated 8 yr before with bark of Femminello Fior d'arancio lemon.

The Parson's Special mandarin trees were kept for one year in a greenhouse at 27-32C and later transferred to the field. Seven months after inoculation they showed severe gum deposition and stunting.

As positive control we used isolates Ca 904 multiplied on Marsh grapefruit, and Ca 908 on Howell

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grapefruit. All trees were grafted on Volkamer lemon rootstock and were planted in an experimental field near Catania.

Nucleic acid extraction. Extraction and partial purification of viroids was carried out according to Lopez-Herrera *et al.* (8) with some modifications. Ten grams of leaves of different ages, or cortex samples collected from young stems in spring, summer, autumn, and winter, were powdered in liquid nitrogen and stored at -75°C for later use. The powder was mixed with phenol, Tris HCl buffer, SDS, EDTA and 2-mercaptoethanol and gently shaken for 30 min. Samples were centrifuged at low speed for 20 min and the aqueous phase was removed, adjusted to a final volume of 30 ml in STE buffer and made to contain 35% ethanol. After addition of cellulose powder (CF11, Whatman), the mixture was gently shaken at room temperature for 1 hr, then transferred to a column and washed with STE buffer containing 35% absolute ethanol. Nucleic acids were eluted with STE buffer, precipitated with absolute ethanol plus 1/10 volume of 3M sodium acetate overnight at -20°C and then centrifuged.

Healthy tissues were subjected to the same procedure.

Polyacrylamide gel electrophoresis and molecular hybridization analysis. Partially purified nucleic acids were subjected to two-cycle polyacrylamide slab gel electrophoresis (dPAGE) following the method of La Rosa *et al.* (7) and Albanese *et al.* (1). To enhance separation between different viroids the discontinuous-pH electrophoretic system suggested by Rivera-Bustamante *et al.* (10) was employed in the second direction in some of the analyses.

RNA CEVd and RNA ASBVd (Avocado sunblotch viroid) were used as markers in all electrophoretic analyses.

Gels were stained with silver. When RNA viroids were recovered for biological tests, gels were stained by ethidium bromide.

Blotting of nucleic acids from gel to nylon membranes and hybridization analysis were conducted as described by Albanese *et al.* (1). CEVd and HSVd-cDNA probes were used in molecular hybridization tests.

Inoculation of herbaceous hosts. Gel pieces containing the different viroid RNAs were excised and then homogenized by mortar and pestle. RNA viroid was extracted by dilution in STE buffer and precipitated with absolute ethanol plus sodium acetate. Concentrated RNAs were inoculated into *Gynura sarmantosa* stems and Rutgers tomato seedlings by razor slashing. All inoculated plants were pruned 4 weeks after inoculation. The second growth flush of tissues was collected and the nucleic acids extracted as reported before and analyzed by dPAGE.

RESULTS

In dPAGE analysis all tested trees showed circular RNAs belonging to citrus viroid group II and/or III. Ca 904 and Ca 908 showed only CCaVd RNA, whereas Femminello Fior d'arancio showed two bands: one slightly slower, and the second slightly faster than the CCaVd RNA associated with Ca 904 and Ca 908. Femminello Continella possessed only one band of similar size to the fastest present in Femminello Fior d'arancio. Moreover CEVd was present in both clones of lemon (fig. 1).

Extracts from Parson's Special mandarin showed the same electrophoretic pattern as Femminello Fior d'arancio lemon.

PAGE analysis using a discontinuous-pH system showed that the Ca 904 isolate possessed an additional band of the same size as the fastest of the two lemons (fig. 2).

CEVd-cDNA probe hybridized only with CEVd (table 1), while positive reactions were obtained between the HSVd-cDNA probe and CVd II

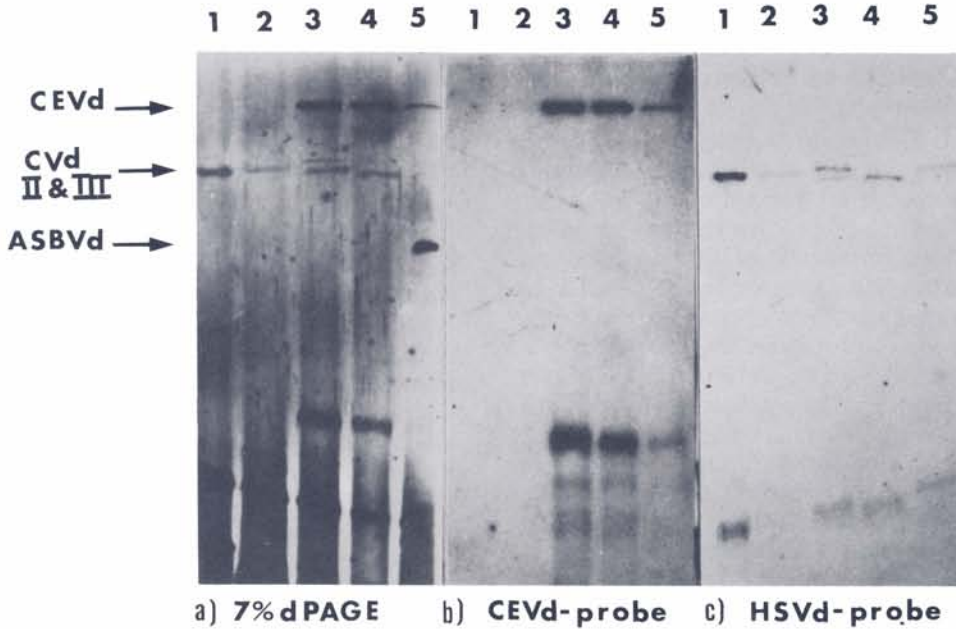


Fig. 1. a) Silver-stained 7% polyacrylamide gel after bidirectional electrophoresis with the second direction run performed under denaturing conditions; b) autoradiograph of a transblot from a gel identical to a) after hybridization with CEVd-specific cDNA probe; c) same, but hybridization with an HSVd-specific cDNA probe.

Samples were: 1) Marsh grapefruit affected by Ca904 California isolate; 2) Howell grapefruit affected by Ca908 California isolate; 3) F.F.D.A. lemon and 4) F.C. lemon both affected by Sicilian isolates of cachexia; 5) purified ASBVd and Navelina 8 sweet orange infected with CEVd and CVdII used as marker.

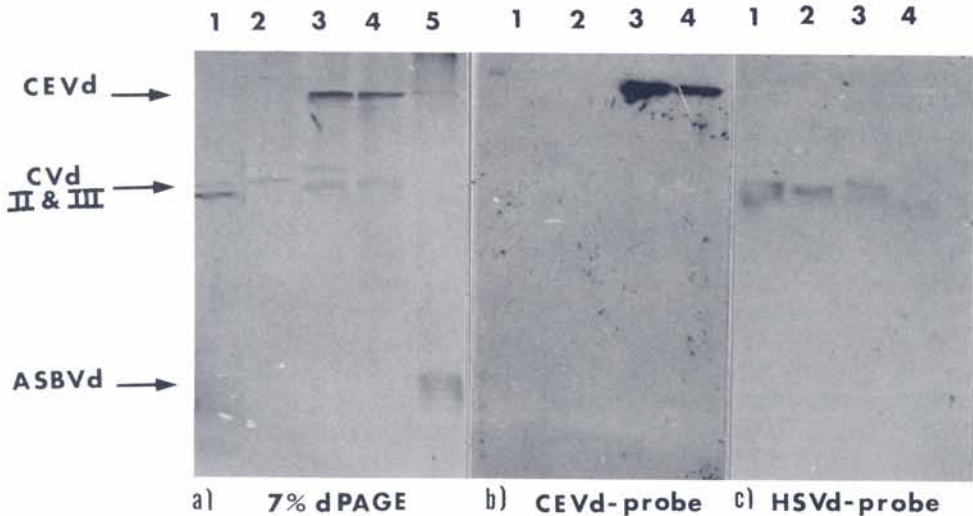


Fig. 2. a) Silver-stained 7% polyacrylamide gel after bidirectional electrophoresis with the second direction run performed under denaturing conditions in a discontinuous-pH system; b) autoradiograph of a transblot from a gel identical to a) after hybridization with CEVd-specific cDNA probe; c) same, but hybridization with an HSVd-specific cDNA probe.

Samples were: 1) Marsh grapefruit affected by Ca904 California isolate; 2) Howell grapefruit affected by Ca908 California isolate; 3) F.F.D.A. lemon and 4) F.C. lemon both affected by Sicilian isolates of cachexia; 5) purified ASBVd and Navelina 8 sweet orange infected with CEVd and CVdII used as marker.

TABLE 1
VIROID INFECTIONS IN DIFFERENT CITRUS HOSTS AND RESULTS OF
cDNA HYBRIDIZATION WITH CEVd AND HSVd PROBES

Citrus hosts and CVd isolates	Viroid infections ^z			cDNA hybridization	
	CEVd	CCaVd	Others	CEVd	HSVd
Howell grapefruit (Ca908)	-	+	-	-	+
Marsh grapefruit (Ca904)	-	+	+	-	+
Femminello Fior d'arancio lemon (F.F.D.A.)	+	+	+	+	+
Femminello Continella lemon (F.C.)	+	+	-	+	+
Parson's Special mandarin (F.F.D.A inoculated)	+	+	+	+	+

^zBased on PAGE analyses and biological tests on Etrog citron and Parson's Special mandarin trees.

and, in contrast to our own previous results (1), also CVd III.

Analysis of RNA extracted from herbaceous hosts inoculated with the bands referable to CEVd and CVd group II and III by dPAGE showed only CEVd RNA.

Results presented in table 2 show that it was possible to detect CCaVd and other viroids of similar size to CCaVd in May, June, July, September, October and November using only 10 g of field collected bark. In March and sometimes in January 20 g of bark tissues were required for positive results. Under our conditions best results were obtained when bark instead of young leaves were used. Temperatures in the field exceed 24C from May until September corresponding to the optimum period for viroid detection (fig. 3).

DISCUSSION

Our results show that CCaVd can be detected by dPAGE in 10 g of field samples collected in summer and autumn, while in winter up to 20 g of tissue may be required.

Under these conditions best results were obtained using bark or young leaves. High temperatures recorded in Sicily in 1989 for the summer months possibly increased CVd replication and consequently their detection.

In spite of the fact that all tested samples indexed positive for cachexia in biological assays on Parson's Special mandarin (4), they did not show the same electrophoretic patterns. The California isolates both possessed CCaVd, with Ca 904 also containing CVd IIIb, whereas F.F.D.A. and F.C. both had CVd IIIb and

TABLE 2
RESULTS OF CACHEXIA AND OTHER VIROIDS DETECTION BY PAGE ANALYSIS OF
SAMPLES COLLECTED IN DIFFERENT MONTHS

Source plants (strain)	Months								
	J	F	M	J	J	S	O	N	
Howell grapefruit (Ca908)	+ ^z	- ^y	-	-	+	+	+	+	+
Marsh grapefruit (Ca904)	-	-	-	-	+	+	+	+	+
Femminello Fior d'arancio lemon (F.F.D.A.)	-	-	+	+	+	+	+	+	+
Femminello Continella lemon (F.C.)	-	-	+	+	+	+	+	+	+
Parson's Special mandarin ^x	-	-	+	+	+	+	+	+	+

^z+ = All viroids present on tree were detected by PAGE analysis (see table 1).

^y- = All viroids present on tree were not detected by PAGE analysis.

^xParson's Special mandarin trees were 10 yr old and have been inoculated 8 yr ago with bark of Femminello Fior d'arancio lemon (F.F.D.A.).

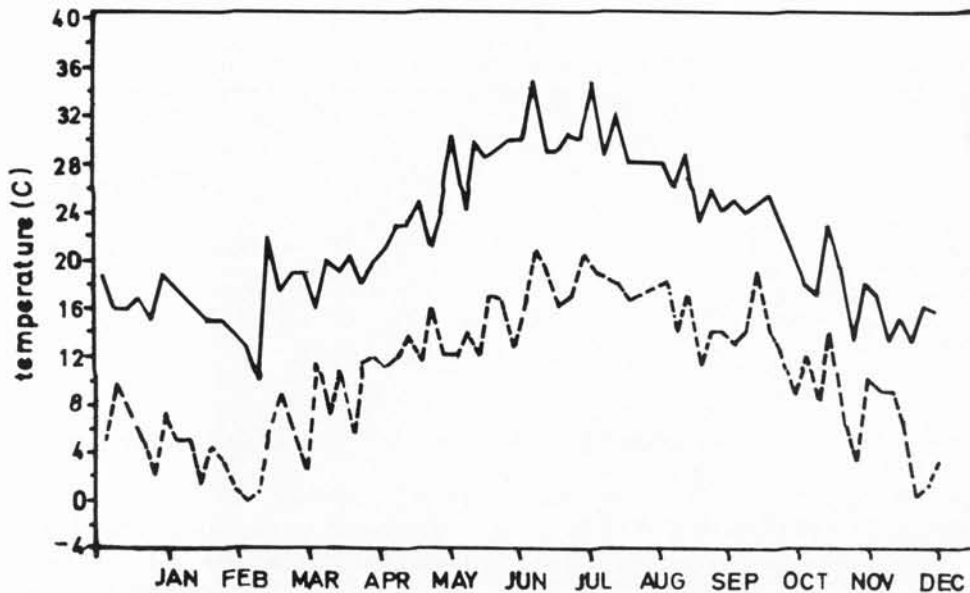


Fig. 3. Maximum and minimum temperatures recorded in 1989 in the experimental field near Catania.

F.F.D.A. also possessing CVd IIa. These results suggest that CVd IIIb could be responsible for some symptoms attributed to cachexia disease.

Positive hybridization of CEVd with CEVd-probe was an expected result, whereas hybridization with HSVd probe gave unexpected findings with both CVd II and III RNAs present in tested samples hybridizing with the HSVd probe. Positive reaction of CVd II with HSVd-probe has been reported previously (1, 5), but hybridization of CVd III with HSVd-probe has not. It is possible that this CVd IIIb isolate hides a comigrating RNA of II group which is responsible for the positive hybridization with HSVd-probe. Further investigations into the citrus viroid complex are needed.

Inoculations to *Gynura* and tomato confirm that CCaVd belongs to group II, and are in agreement with the results of Semancik *et al.* (14). In fact, for these researchers, the viroids of group II are unable to replicate in *Gynura* and tomato, but only in cucumber seedling of the variety Suyu. These results are not surpris-

ing. In fact, there is evidence suggesting that HSVd, first found in Japan in grapevine (9), is actually a latent viroid of this species distributed worldwide, and was found later by Diener *et al.* (5) in commercial citrus trees and by Puchta *et al.* (9) in Etrog citron plants inoculated with a graft transmissible dwarfing agent (GTDA).

During experiments with old clones of lemon we have found other citrus viroids. This is probably the result of topworking of trees, a common practice in Italy.

More research is needed to catalogue citrus viroids and to clarify the role of these small molecules on citrus and other trees.

Until now, the only specific names for members of the citrus viroid family that seem appropriate are the citrus exocortis viroid (CEVd) and the citrus cachexia viroid (CCaVd), now very likely a strain of hop stunt viroid (HSVd).

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