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A Viroid Different from Citrus Exocortis Viroid Found in Commercial Citrus in Sicily

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ABSTRACT. A low molecular weight RNA, named citrus "B" viroid (CBV), has been detected alone or associated with citrus exocortis viroid (CEV) in many commercial citrus species and varieties. The electrophoretic mobility of CBV on 5% polyacrylamide gels under denaturing conditions is between those of CEV and the fast form of coconut cadang cadang viroid (CCCV) RNA1. CBV replicates in zucchini squash, a common host for CEV, but not in ten other herbaceous hosts of CEV. Citrus nucleic acid extracts containing CBV were reacted with full-length CEV-, potato spindle tuber viroid (PSTV)-cDNA probes and with CEV-, PSTV-, tomato apical stunt viroid (TASV)- and tomato planta macho viroid (TPMV)-RNA probes. CBV did not hybridize with any of the probes. Inoculations of partially purified CBV onto different citrus indicator plants gave variable epinasty on Etrog citron, but no symptoms in other indicators.

Index words. PAGE, molecular hybridization.

Detection of citrus exocortis viroid (CEV) by polyacrylamide gel electrophoretic (PAGE) analysis of citrus nucleic acid extracts has been widely demonstrated (1, 3, 4). We routinely use this method of CEV diagnosis because it is suitable for detecting severe as well as mild isolates and requires only a small amount (1-3 g) of citrus tissue from the greenhouse or from the field (8).

A new viroid, which we named citrus "B" viroid to distinguish it from CEV, has been detected recently in many Sicilian commercial citrus trees (2). Many citrus viroids other than CEV also have been found by different authors in other countries (6, 10). We report here studies to determine whether CBV is related to CEV or may be associated with other citrus diseases caused by unknown pathogenic agents.

MATERIALS AND METHODS

Inoculum source. Tissue samples taken from citrus trees located in different citrus areas were used. Source plants had been previously indexed for psorosis, cachexia (xyloporosis) and CEV by routine tests (2)

and were propagated on Volkamer lemon rootstock or graft inoculated onto Arizona 861-S-1 citron and grown in the greenhouse at 30-37 C.

A severe CEV isolate (PV-194) from the American Type Culture Collection (ATCC) was inoculated by stem-slashing into Arizona 861-S-1 citron and into Volkamer lemon as a control.

Electrophoretic tests. Young bark of shoots collected in the field (during spring or autumn) or in the greenhouse was used to extract nucleic acids from citrus. These were obtained by a phenol-chloroform extraction and 2M LiCl-fractionation (1). Nucleic acids from herbaceous plants were extracted by the method of Macquaire, *et al.* (9). Extracts were electrophoresed in bidirectional 5% polyacrylamide gels with second direction electrophoresis under denaturing conditions (11). The fast form of coconut cadang cadang viroid (CCCV) RNA1 was coelectrophoresed as a marker. Silver stain (Bio-rad, Richmond, California) protein protocol, was used to develop slabs (4).

Biological tests. CEV and CBV RNAs were separately recovered from denaturing gels (dPAGE) by cutting out the bands corresponding to circular forms and grinding the gel in a mortar and pestle in 0.05 M

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glycine, 0.03 M K_2HPO_4 (pH 9.2) buffer. Viroids were stem-slash inoculated into Arizona 861-S-1 citron and Parson's Special mandarin.

Nucleic acids extracted from CBV- and CEV-infected citrus trees were dissolved in 0.04 M K_2HPO_4 (pH 8.0) buffer and inoculated by stem-slashing into herbaceous hosts listed in table 2 and by rubbing onto carborundum-dusted young leaves of *Gynura aurantiaca* D.C.

CEV (PV-194) was used as a control in every inoculation.

Infection was checked weekly by observation of symptoms and monthly by electrophoretic analysis.

Molecular hybridization. To prepare ^{32}P -labeled DNA and RNA probes, double-stranded cDNAs of CEV-J variant "d" (13), potato spindle tuber viroid (PSTV) (5), tomato apical stunt viroid (TASV) (7), and tomato planta macho viroid (TPMV) (7) were separately inserted into appropriate restriction endonuclease sites (specific for each viroid) of vector pSP65 and amplified in *Escherichia coli* strain JM83. High specific activity radioactive probes were prepared either by nick-translation using a commercial kit (Bethesda Research Laboratories) or by *in vitro* synthesis of an RNA probe using SP6 RNA polymerase and a linearized DNA template (Promega Biotec, Inc.).

Nucleic acid extracts from citrus, which in PAGE analysis disclosed CEV, CEV + CBV, or CBV, and extracts from tomato which had been inoculated with PSTV or CEV + CBV were denatured with 4 M formaldehyde and were used undiluted or diluted three-, ten- and one-hundred fold with 7.5X SSC buffer (15 mM NaCl, 1.5 mM sodium citrate).

In dot blot tests, samples were pipetted (2 μ l/dilution) onto a treated nitrocellulose membrane (12). In northern blot analysis, extracts were transferred onto membranes after electrophoresis on 1.5% agarose. Nitrocellulose membranes with bound extracts were then baked 2 h at 80 C *in vacuo*.

The prehybridization reaction was performed using 1 ml of buffer with 40% (v/v) formamide, 0.18 M NaCl, 10 mM sodium cacodylate, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and calf thymus DNA (300 μ g/ml) at pH 7.0 per 35 cm² of membrane area at 55 C for 30 min to 1 h. Dextran sulfate was then added to 10% (w/v) and the membranes further incubated for 30 min to 1 h. Hybridization was

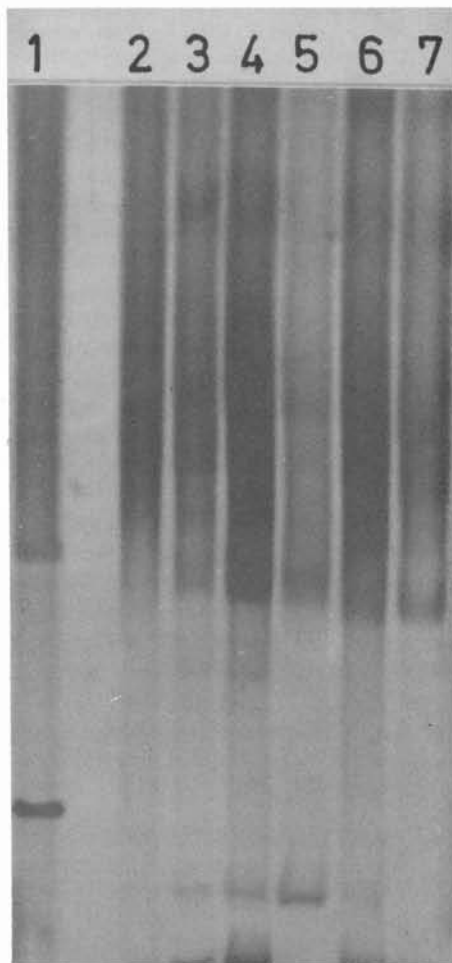


Fig. 1. Second direction polyacrylamide gel (5%) electrophoresis (PAGE) (upwards migration under denaturing conditions and staining with silver), of nucleic acids extracted from: 1—coconut cadang-cadang viroid (CCCV) RNA 1-fast-infected coconut; 2, 3, 4 and 6—citrus exocortis viroid (CEV)—and citrus "B" viroid (CBV)-infected citrus; 5—CBV-infected citrus; 7—CEV-infected citrus.

performed for 18 h at 42 or 55 C in the presence of 10% dextran sulfate (14) and ^{32}P -labeled probes ($1-2 \times 10^6$ cpm/ml for DNA probes and $0.3-0.5 \times 10^6$ cpm/ml for RNA probes). The membranes were then washed at 55 C with three changes (10 min/change) of buffer (0.36 M NaCl, 0.02 M Tris, 0.1% SDS, pH 7.0) and then with three changes of 0.1X of the above buffer. An additional stringent wash at 65 C with 3 changes (20 min/change) of 0.1X SSC with 0.1% SDS was made to the membranes hybridized with RNA-probes.

Membranes hybridized with RNA-probes were then treated with RNase A (1 $\mu\text{g}/\text{ml}$) in 2X SSC buffer at room temperature for 15 min and then rinsed at 50 C for 30 min in 0.1X SSC with 0.1% SDS.

Autoradiography was carried out overnight at -70 C with Kodak XAR-5 film and intensifying screen.

RESULTS

Electrophoretic tests and comparison with bioassays of source plants. CBV electrophoretic mobility, between CEV and fast form of

CCCV RNA1 (fig. 1), reveals a molecular size of about 308 nucleotides. Detection frequency of CBV and/or CEV in tested citrus plants by PAGE and by bioassay on Arizona 861-S-1 citron is reported in table 1. When PAGE revealed only CBV, the reaction of Arizona 861-S-1 citron was invariably mild, whereas when PAGE revealed CEV alone or CEV and CBV, symptoms on citron were always severe.

Biological tests. Inoculations of viroid preparations eluted from dPAGE induced symptoms. CEV infection of Arizona 861-S-1 citron resulted in severe stunting, leaf curling, and veinal necrosis, whereas CBV infection resulted only in mild stunting and variable epinasty (fig. 2) 1 month after inoculation. PAGE analysis of these tissues disclosed the presence of a viroid RNA with the mobility of the RNA used for inoculation.

Extracts from source trees were also inoculated on Parson's Special mandarin. One year after inoculation all test plants were negative for the browning reaction for cachexia (xyloporosis).

TABLE 1
POLYACRYLAMIDE GEL (5%) ELECTROPHORETIC (PAGE) ANALYSIS AND BIOASSAY RESULTS ON CITRON FOR SOURCE PLANTS CONTAINING CITRUS EXOCORTIS VIROID (CEV) AND CITRUS "B" VIROID (CBV)

Source plants	No. of PAGE tests detecting			Total no. of PAGE tests	Pathogenicity on Arizona 861-S-1 citron
	CEV	CBV	CEV + CBV		
Citron ^z	15	0	0	15	Severe
Volkamer lemon ^z	5	0	0	5	Severe
Navelina 1 sweet orange	0	11	0	11	Mild
Navelina 8 sweet orange	5	3	9	17	Severe
Vaniglia sweet orange	0	5	0	5	Mild
Continella Fem. lemon	2	0	1	3	Severe
Avana mandarin	0	3	0	3	Mild
Murcott tangor	0	3	0	3	Mild
Comune 1 clementine	0	4	0	4	Mild
Comune 2 clementine	2	0	13	15	Severe
Comune 3 clementine	1	3	7	11	Severe
Star Ruby grapefruit	0	0	3	3	Severe
Fantastico bergamot	0	0	3	3	Severe
Volkamer lemon	0	3	0	3	Mild

^zInoculated with CEV isolate PV-194 ATCC.



Fig. 2. Arizona 861-S-1 citrons (from left to right) inoculated with citrus exocortis viroid (CEV), citrus "B" viroid (CBV) (gel band), and healthy.

TABLE 2
BIOLOGICAL REACTION AND POLYACRYLAMIDE GEL (5%) ELECTROPHORETIC ANALYSIS OF HERBACEOUS HOSTS 60-70 DAYS AFTER INOCULATION WITH NUCLEIC ACID EXTRACTS CONTAINING CITRUS EXOCORTIS VIROID (CEV) AND CITRUS "B" VIROID (CBV)

Host	Symptoms ^z	PAGE ^z	
		CEV	CBV
<i>Aster grandiflorus</i>	—	+	—
<i>Capsicum annuum</i> 'Yolo Wonder'	—	+	—
<i>Chrysanthemum morifolium</i> 'White Spider'	—	+	—
<i>Cucurbita pepo</i> Italica	+	+	+
<i>Dahlia variabilis</i>	—	+	—
<i>Gynura aurantiaca</i>	+	+	—
<i>Lycopersicon esculentum</i> 'Rutgers'	+	+	—
<i>Ocimum basilicum</i>	—	+	—
<i>Petunia hybrida</i>	—	+	—
<i>Solanum melongena</i> 'Long Purple'	—	+	—
<i>Zinnia elegans</i>	—	+	—

^z+ = presence or — = absence of symptoms or viroid band.

Among herbaceous hosts inoculated with citrus nucleic acid extracts containing CEV and CBV, only Rutgers tomato, *Gynura aurantiaca* and zucchini squash showed epinasty and a mild stunting. CEV was recovered by PAGE analysis 60-70 days after inoculation from all tested species, whereas CBV could be detected only in zucchini squash extracts (table 2).

Molecular hybridization. A total of 63 citrus extracts classified into three groups (CEV, CEV + CBV, and CBV) according to PAGE analysis were tested by dot-blot hybridization with CEV-, PSTV-, TASV-, and TPMV-RNA probes. The number of hybridized extracts of the total spotted per group and their degree of hybridization are reported in table 3. Almost all citrus extracts of CEV and CEV + CBV groups hybridized with every probe used. The lack of reaction of some samples may be attributable to handling errors. Of 26 CBV extracts tested with all of the probes, only one or two hybridized. These positive reactions may be CEV which was not detected by PAGE analysis in those few extracts. The observed differences in hybridization intensity are consistent with the degree of homology among the respective viroids.

Results of northern blot hybridization carried out on some extracts with PSTV- and CEV-cDNA probes agree with those of dot blot assays (fig. 3)

TABLE 3
HYBRIDIZATION WITH RNA PROBES OF CITRUS EXTRACTS SHOWING CITRUS EXOCORTIS VIROID (CEV), CEV PLUS CITRUS "B" VIROID (CBV) OR CBV ONLY IN POLYACRYLAMIDE GEL (5%) ELECTROPHORETIC (PAGE) TESTS

Viroids detected by PAGE	RNA probes					
	CEV		TAS + TPMV ^z		PSTV ^z	
	a ^y	b ^y	a	b	a	b
CEV	12/15	+++	4/15	++	2/15	+
CEV + CBV	20/23	+++	14/23	++	3/23	+
CBV	2/26	+++	1/26	++	1/26	+

^zTASV = tomato atypical stunt viroid; TPMV = tomato planta macho viroid; PSTV = potato spindle tuber viroid.

^ya = no. positive of total samples tested; b = degree of hybridization: +++ high; ++ medium; + low.

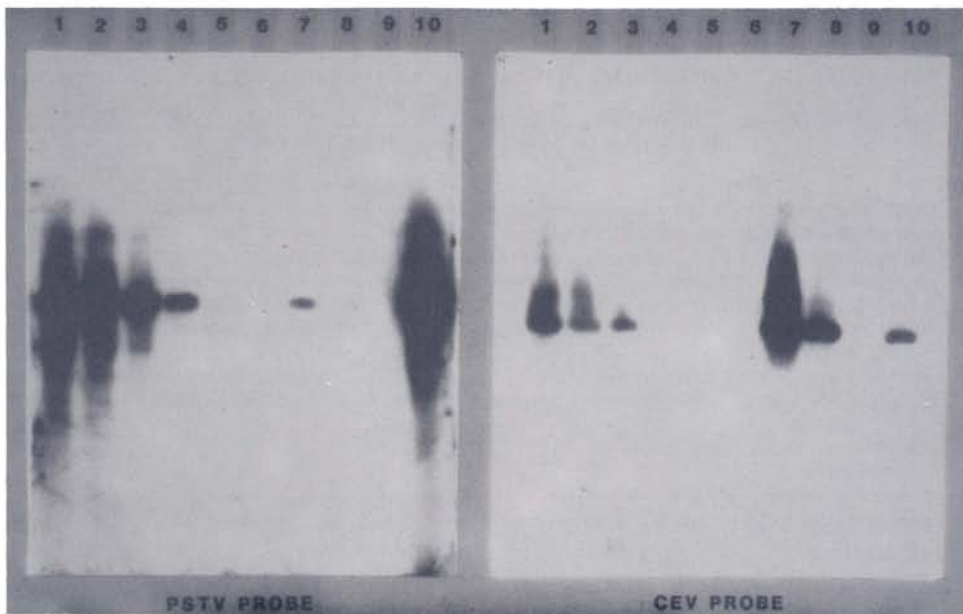


Fig. 3. Autoradiographs of northern blots with nucleic acid extracts of: 1 and 5—potato spindle tuber viroid (PSTV)-inoculated tomato at decreasing dilutions; 6—healthy tomato; 7—citron inoculated with citrus exocortis viroid (CEV) (ATCC PV-194); 8—Navelina 8 sweet orange infected with CEV and citrus "B" viroid (CBV); 9—citron inoculated with Navelina 1 sweet orange infected with CBV; 10—gel eluted PSTV. Hybridization with cDNA probes of PSTV and CEV.

and verify the absence of any reactions between CBV and all of the probes utilized.

Extracts of tomato plants inoculated with citrus extracts containing CEV + CBV hybridized with the PSTV probe better than with CEV probe (fig. 4). To eliminate spurious hybridization results with RNA probes, it was essential to treat membranes with RNase prior to autoradiography (fig. 5).

DISCUSSION

The electrophoretic mobility of CBV, its pathogenicity on Arizona 861-S-1 citron, and lack of replication on most herbaceous hosts of CEV suggest that it is a viroid physically and biologically distinct from CEV.

Comparison between CBV and other new citrus viroids (6, 10) has been attempted. Mild stunting and variable epinasty induced by CBV on Arizona 861-S-1 citron seems to be

very similar to symptoms induced by citron variable viroid (CVaV) (10).

Moreover, CBV electrophoretic mobility suggests a molecular size within the range (311 to 335 nucleotides) reported for RNAs I, II and III (6). However, electrophoretic conditions and comigrated markers in that work were different from ours and therefore results are not comparable. More experiments must be made to demonstrate whether any of the newly described citrus viroids are similar to CBV and/or if one of them is identical with CBV.

The lack of hybridization of extracts containing CBV with any of the probes used indicates that this viroid is different from the PSTV group viroids. Extracts from citrus trees infected with CEV and CEV + CBV hybridized, as expected, more strongly with the CEV probe than with the PSTV probe (fig. 3, 4, 5), but extracts from tomatoes that had been inoculated with citrus nucleic

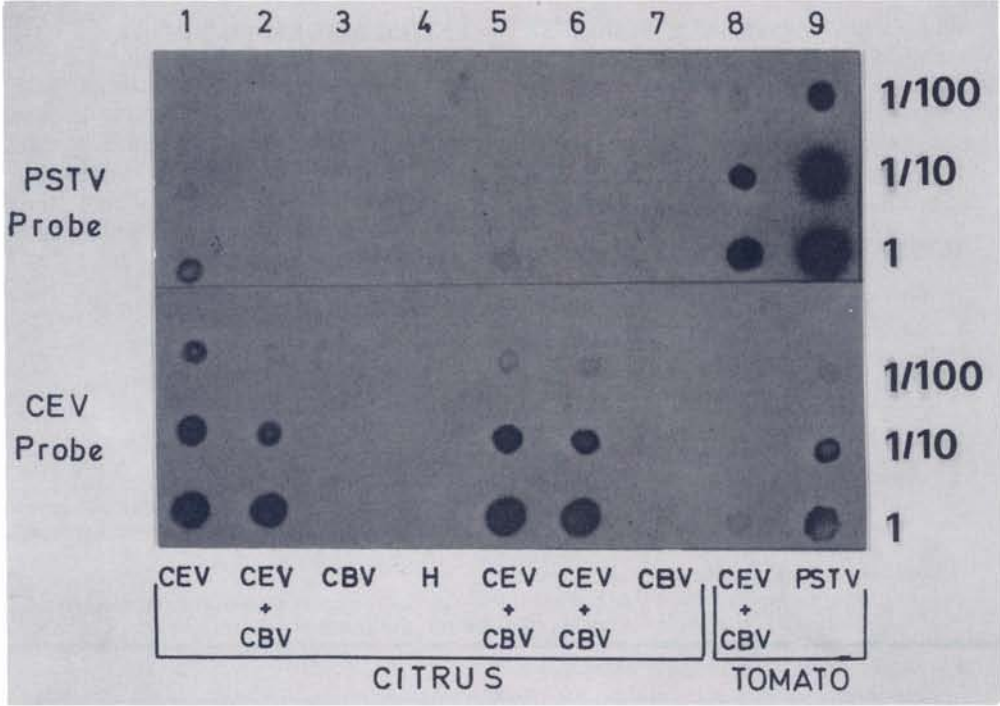


Fig. 4. Autoradiographs of nitrocellulose dot blots with nucleic acid extracted from: 1—citrus exocortis viroid (CEV)-infected citrus; 2, 5 and 6—CEV and citrus “B” viroid (CBV)-infected citrus; 3 and 7—CBV-infected citrus; 4—healthy citrus; 8—CEV+CBV and 9—potato spindle tuber viroid (PSTV)-inoculated tomato. Hybridization with PSTV- and CEV-cDNA probes.

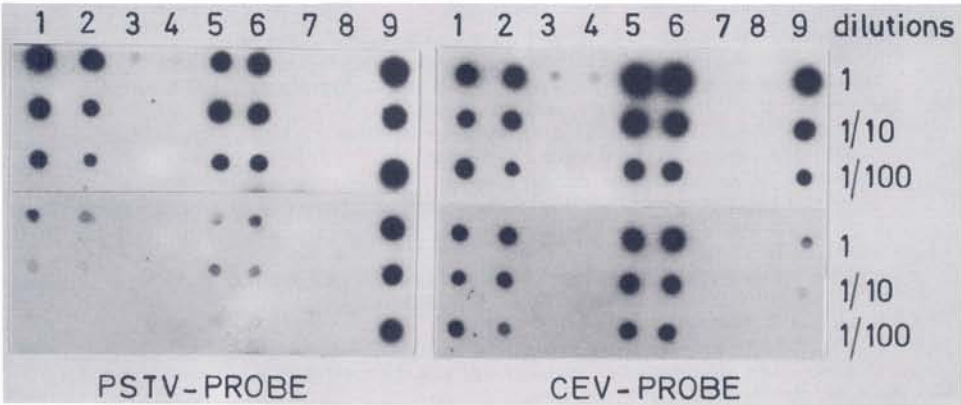


Fig. 5. Autoradiographs of nitrocellulose dot blots with nucleic acid extracted from: 1—citrus exocortis viroid (CEV)-infected citrus; 2, 5 and 6—CEV and citrus “B” viroid (CBV)-infected citrus; 3, 7 and 8—CBV-infected citrus; 4—healthy citrus; 9—potato spindle tuber viroid (PSTV)-infected tomato. Hybridization with PSTV- and CEV-RNA probes, before (above) and after (below) RNase treatment.

acid extracts containing both CEV and CBV hybridized more strongly with the PSTV probe than the CEV probe (fig. 4). This unexpected finding remains to be explained.

Studies are in progress to determine the CBV nucleotide sequence.

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NOTE ADDED IN PROOF

Nucleic acid hybridization experiments with a cDNA probe to hop stunt viroid (kindly supplied by Dr. E. Shikata, Hokkaido University, Sapporo, Japan) have shown that CBV is a strain of this viroid (manuscript in preparation).

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