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Title

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Permalink

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Journal

International Organization of Citrus Virologists Conference Proceedings
(1957-2010), 10(10)

ISSN

2313-5123

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Publication Date

1988

DOI

10.5070/C55h41z47c

Peer reviewed

Suitability of Nucleic Acid Analysis to Diagnose Viroid Infections in Citrus

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ABSTRACT. The suitability of polyacrylamide gel electrophoresis (PAGE) analysis of nucleic acid extracts to diagnose viroid infection in citrus was investigated. Samples obtained from field trees and/or their propagations in the greenhouse were analyzed all year. The best period to assay field samples was from March to November. The bark of green twigs appeared more suitable than leaves. Infected samples showed one to six circular forms of viroidlike RNAs: one of these was identified as citrus exocortis viroid (CEV), another was partially characterized and named citrus B viroid (CBV), others have not yet been characterized. PAGE analysis appears faster and more accurate than assay on Etrog citron, allowing identification and differentiation of many viroid infections at once.

Index words. PAGE, viroid, citrus.

Polyacrylamide gel electrophoretic (PAGE) analysis of citrus nucleic acid extracts has already been demonstrated for citrus exocortis viroid (CEV) diagnosis (1, 4, 5). Recently, this method allowed detection of a new viroid, named citrus B viroid (CBV), in many citrus species and varieties in Sicily (2, 3). Another pathogenic RNA, named citron variable viroid (CVaV) (7), and additional viroidlike RNAs (6) also have been identified in citron by PAGE.

The suitability of PAGE to detect citrus viroids in the routine indexing of different citrus species grown either in the field or in the greenhouse was further investigated in the present paper.

MATERIALS AND METHODS

Plant materials. Different citrus species and varieties grown in the field and affected by virus and virus-like diseases were tested by PAGE analysis. Many of them were graft-propagated on Volkamer lemon and grown in a greenhouse at 30 ± 2 C. Shoot-tip-grafted citrus plants and their source plants, provided by Istituto Coltivazione Arborea, Università di Catania, and Istituto Sperimentale per l'Agrumicoltura, Acireale, were also screened. All plants were previously bioassayed on Etrog citron. As a positive control some indicator plants were slash in-

oculated with a severe strain of CEV (PV- 194 from the American Type Culture Collection).

Samples from field trees were collected monthly. Young, expanded leaves were used (1); when they were not available young, green bark was collected.

PAGE analysis. Citrus nucleic acid extraction was carried out using 1-5 g of tissue (young leaves and/or green bark), as previously reported (1).

Electrophoretic analysis of extracts (either bidirectional or bidimensional) was performed in 5% polyacrylamide gel slabs as described by Schumacher *et al.* (8). Samples were electrophoresed in the first direction (at 225V, 20C, for 2 h); the second run was made under denaturing conditions (upward direction, 8M urea, 250 V, 50C, for 3 h). Coconut cadang-cadang viroid (CCCV) fast RNA-1 and citrus exocortis viroid (CEV) were used as markers.

The slabs were stained with silver (BIO-RAD, Richmond, California) (5).

RESULTS

Citrus species and varieties tested by PAGE gave different RNA patterns (table 1). Six different bands were observed in the analysis and up to 4 bands were detected in a single sample. CEV and CBV were found in

TABLE 1
FREQUENCY OF VIROID AND VIROIDLIKE RNAs FOUND BY PAGE ANALYSIS IN DIFFERENT CITRUS SPECIES TESTED POSITIVE ON ETROG CITRON FOR EXOCORTIS SYMPTOMS

Donor species	No. of samples tested	No. of samples showing viroid bands ^z			Viroidlike RNAs bands			
		CEV	CBV	CEV + CBV	a	b	c	d
Sweet orange	59	7	12	40	—	—	1	5
Lemon	31	8	1	22	—	2	—	—
Mandarins and relatives	19	1	8	10	1	4	6	1
Sour orange ^y	14	0	0	14	—	3	—	—
Bergamot	6	0	0	6	—	2	1	—
Grapefruit	5	0	2	3	—	2	—	—
Volkamer lemon	23	5	5	13	—	4	—	—
Citron ^x	40	40	0	0	—	—	—	—
Citron ^y	29	6	17	6	—	4	1	—

^zCEV = citrus exocortis viroid; CBV = citrus B viroid.

^yPlants inoculated with different sources.

^xPlants inoculated with CEV PV-194.

every citrus plant, either alone, or associated with others. Other bands were found in addition to CEV and/or CBV in different combinations (table 1). All of them had electrophoretic mobilities in the zone between circular viroid molecules of CEV and CCCV RNA-1 fast. CBV migrated between CEV and CCCV RNA-1 fast, revealing a molecular size of about 308 nucleotide residues. Other additional bands, named a-d viroidlike RNAs, migrated as follows: a and b migrated between CEV and CBV; and c and d migrated between CBV and CCCV RNA-1 fast (fig. 1). Shoot-tip-grafted plants were viroid-free.

Donor plants that induced severe symptoms on Etrog citron always showed the CEV band alone or associated with CBV or other viroidlike RNAs in PAGE analysis; mild symptoms on citron indicators were associated with CBV and other viroidlike RNAs except CEV. Etrog citron, experimentally inoculated with the severe strain of CEV, displayed a unique CEV-RNA circular form.

PAGE analysis of samples collected from the field showed that bark is more suitable than leaves and that it allowed detection of viroids during

all seasons except winter (table 2). During the growing season (March-November), very good results were obtained and up to 100% of infected samples were indexed positively.

No difference in PAGE was found between leaves and bark collected in the greenhouse; both were suitable for the test, but extraction was easier from bark than from leaves.

DISCUSSION

The PAGE procedure described allowed reliable detection not only of known CEV, but also of other citrus viroids (CBV and viroidlike RNAs a-d).

Electrophoretic mobility of new viroids in PAGE analysis under denaturing conditions showed they have different molecular weights. Biological and hybridization tests have shown that CBV is different from CEV (3). The role of the other viroidlike RNAs in the exocortis syndrome remains to be investigated.

The method allowed processing of many samples in a short time (3-4 days); but its main advantage was to test viroid infections from any citrus species and variety using the bark samples collected directly from the

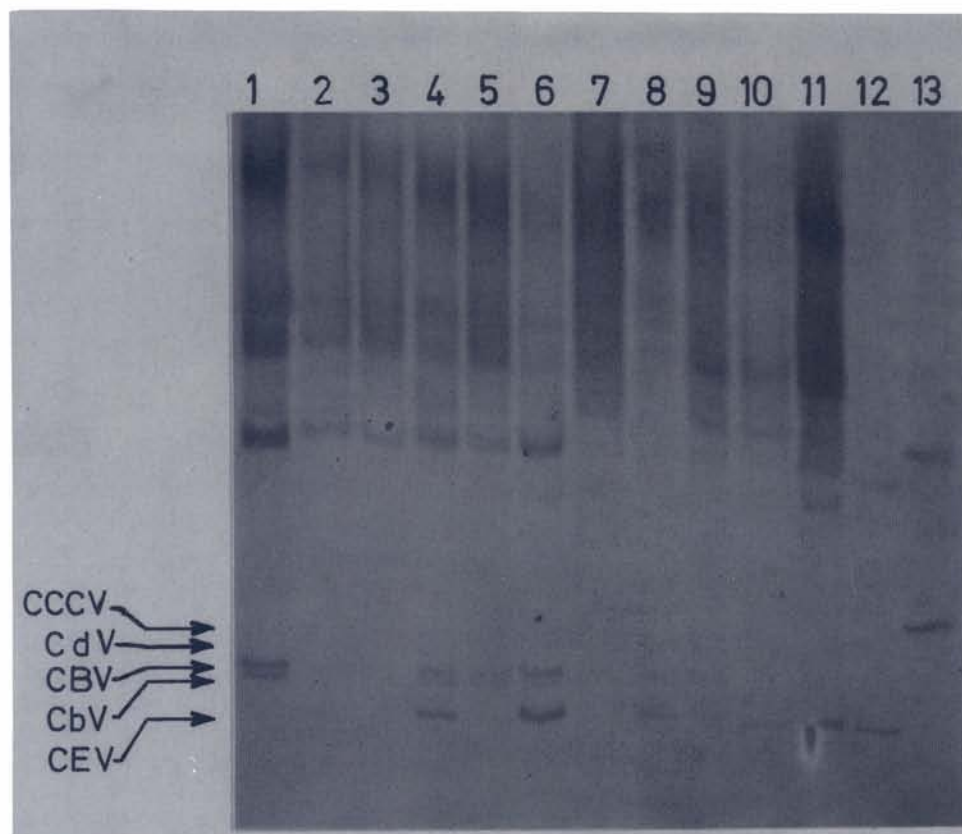


Fig. 1. Second direction PAGE under denaturing conditions (8M urea) of nucleic acids extract from: lanes 1 and 5, CBV- and viroidlike RNA-b-affected citrus; lanes 2, 3 and 7, shoot-tip grafted citrus; lanes 4 and 6, CEV, CBV and viroidlike RNA-b-affected citrus; lane 8, CEV and viroidlike RNA-d-affected citrus; lane 9, CBV-affected citrus; lanes 10-12, CEV-affected citrus; and lane 13, CCCV fast RNA-1. Gel was silver stained. Migration is upward.

TABLE 2
RESULTS OF VIROID AND VIROIDLIKE
RNA DETECTION BY PAGE ANALYSIS
IN CITRUS LEAVES AND BARK COL-
LECTED IN THE FIELD (IN SPRING)

Species and varieties	Viroids detected in: ^z	
	Leaves	Bark
Ovale sweet orange	—	CEV + CBV
Star Ruby grapefruit	—	CEV + CBV
Duncan grapefruit	—	CBV + b
Femminello Continella	—	CEV + CBV
lemon	—	CBV + b
Ortanique mandarin	—	CBV
Etrog citron	—	CBV
(slash inoculated)	—	CBV
Etrog citron	—	CBV
(bud inoculated)	—	CBV

^zCEV = Citrus exocortis viroid; CBV = Citrus B viroid; b = b viroidlike RNA.

field. Hence, compared to the traditional tests on Etrog citron, PAGE appears faster and more accurate because it allows the identification of the particular viroid responsible for the infection. Moreover, it appears particularly suitable for special purposes such as quarantine programs and indexing of small plants obtained by shoot-tip grafting.

ACKNOWLEDGMENT

This work was supported by CNR, Italy. Special grant IPRA. Sub-project No. 1, paper No. 1423.

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