A New Indexing Method for Cachexia

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ABSTRACT. The utilization of nucleic acid extraction and polyacrylamide gel electrophoresis as an alternative to the Parson's Special bioassay for routine indexing of cachexia was evaluated. Three field sources of citrus viroids and three greenhouse controls were graft inoculated on Arizona Etrog citron. Inoculated citrons were periodically analyzed by nucleic acid extraction and sequential gel electrophoresis. The citrus cachexia viroid (CCaV) was detected after 3 months in all the plants tested regardless of the source. Three electrophoresis systems were also compared. The proposed method combines the desirable characteristics of citron as an excellent host for viroid replication and the sensitivity of electrophoresis and silver staining for viroid detection. The method is now routinely used in the Citrus Sanitation Program in Spain.

Index words. citrus cachexia viroid, citrus viroids, xyloporosis.

Biological indexing for citrus cachexia is done by graft inoculation on Parson's Special mandarin on rough lemon rootstock. Inoculated indicators have to be incubated at warm temperatures (28-32 C) for 12-18 months for symptom expression (11). This has been shown to be the most sensitive bioassay for cachexia, and it is being used worldwide. However, this assay has several limitations: a) the long time required for symptom expression; b) high fuel expenses in some temperate zones to heat the greenhouses; c) erratic results even when several indicators and controls are included in each test (10); and d) possible interference with other citrus viroids (8).

A series of tests were conducted to evaluate the usefulness of the nucleic acid extraction and polyacrylamide gel electrophoresis (PAGE) systems developed for viroid studies as an alternative to the Parson's bioassay.

MATERIALS AND METHODS

Viroid sources and inoculation. Six lots of Arizona Etrog citron 861-S1 on Rough lemon rootstock were graft inoculated with three field sources and three single viroid controls. The field sources were Canoneta sweet orange, Mineola tangelo and Marisol clementine. All had been biologically indexed for triesteza, psorosis A, concave gum, vein enation and exocortis. Canoneta sweet orange was found free of tristeza, psororis A and concave gum and positive for exocortis and vein enation; Mineola tangelo as free of tristeza, psorosis A and vein enation and positive for exocortis and concave gum; and Marisol clementine was free of tristeza and vein enation and positive for psorosis A, concave gum and exocortis. The single viroids used as positive controls were CV-IIb or citrus cachexia viroid (CCaV), CV-IIc which has been shown recently to be also a cachexia disease agent (18) and the related viroid CV-IIa. A set of uninoculated controls were also included. The assay was repeated twice. In one assay, the plants were inoculated in October and incubated under natural (short day) light conditions, and in the other assay the plants were inoculated in June and incubated under natural (long day) light conditions. In both cases the inoculated citrons were incubated in a warm greenhouse (28-32 C).

In both assays, tissue samples from separate sets of plants were collected 1, 2, and 3 months after inoculation, for nucleic acid extraction and PAGE. In all instances the tissue was collected from two plants and processed separately.

In some instances, nucleic acid preparations of tomatoes inoculated with PSTV and CSV (kindly provided by T. Candresse from IRNA, Bordeaux and J. Romero from INIA, Madrid) were used. Nucleic acid preparations of mixtures of CV-IIa/CV-IIc were obtained from double inoculated cucumbers.

Nucleic acid extraction. New flush tissue consisting of young leaves and stems (5 g) from inoculated citrons was homogenized in 5 ml of extraction medium (0.4 M Tris.HCl pH 8.9, 1% SDS, 5 mM EDTA pH 7.0, 4% 2-mercaptoethanol) containing 15 ml of water saturated phenol (14). Total nucleic acids were partitioned in 2 M LiCl, and the soluble fraction was concentrated by ethanol precipitation. The final preparation was resuspended in 300 µl of TKM (10 mM Tris, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4).

Polyacrylamide gel electrophoresis (PAGE). In all instances viroid detection was accomplished by subjecting 20 µl aliquots of the nucleic acid preparations (equivalent to 300 mg fresh weight of tissue) to 5% PAGE.

Unless otherwise stated the sequential-PAGE (s-PAGE) system described by Semancik and Harper (15) was used. This electrophoresis procedure has proved invaluable for the characterization of CCaV (16, 17) and citrus viroids (3, 4). The first gel was polymerized in TAE electrophoresis buffer (40 mM Tris, 20 mM sodium acetate. $3H_2O$, 1 mM sodium EDTA, pH 7.2), and subjected to a constant current of 60 mA, at 4 C for 2.5 hr. A segment of the gel defined by the xylene cyanol dye was excised and placed on the top of the second gel containing 8 M urea and polymerized with a pH 6.5 TAE buffer (9). It was subjected to a constant current of 16 mA, at room temperature until the tracking dye reached the bottom of the gel. After completion of the second electrophoresis, the circular forms of the viroids were viewed by silver staining (6).

The two-dimensional-PAGE technique described by Schumaker *et al.* (12) was also evaluated. The first gel was prepared and performed as above. The segment of the gel was placed across the bottom of gel chamber and the second gel containing 8 M urea polymerized above (also with a pH 6.5 TAE buffer). The second PAGE was performed from bottom to top at constant current of 16 mA, at room temperature until the tracking dye reached the top of the gel.

The return-PAGE system described to detect viroids of other crops (13, 19) was also evaluated. The first gel was polymerized in high salt TBE electrophoresis buffer (90 mM Tris, 90 mM boric acid, 2 mM sodium EDTA, pH 8.3), and subjected to a constant current of 46 mA, at room temperature until the xylene cyanol tracking dye reached to bottom of the gel. The second electrophoresis was performed from bottom to top under high temperature conditions (in a incubator at 60 C), by changing the high salt TBE electrophoresis buffer for a low salt TBE buffer (11.25 mM Tris, 11.25 mM boric acid, 0.25 sodium EDTA, pH 8.3), previously heated to 80 C.

Biological indexing. Field isolates and single viroid controls were biologically indexed for cachexia and citrus viroids. The Parson's Special mandarin propagated on rough lemon was used as cachexia indicator (11). Four indicator plants were used in each test and the results were collected 18 months after inoculation. The Arizona Etrog citron 861-S1 was used as indicator of exocortis and citrus viroids. Two citron indicators were used in each test and the results were collected 6 months after inoculation.

RESULTS

Incubation of inoculated citrons for detection of CCaV.

Analysis of nucleic acid extracts from tissue collected one month after inoculation gave poor and variable results, in both assays (October and June inoculations). No viroids were detected from the controls inoculated with single CV-II viroids.

When the tissue was collected two months after inoculation, several viroids were detected from plants inoculated with field sources (Fig. 1A). However, only CV-IIa and CV-IIc were detected in the controls inoculated in October, whereas only CV-IIb was detected in the controls inoculated in June

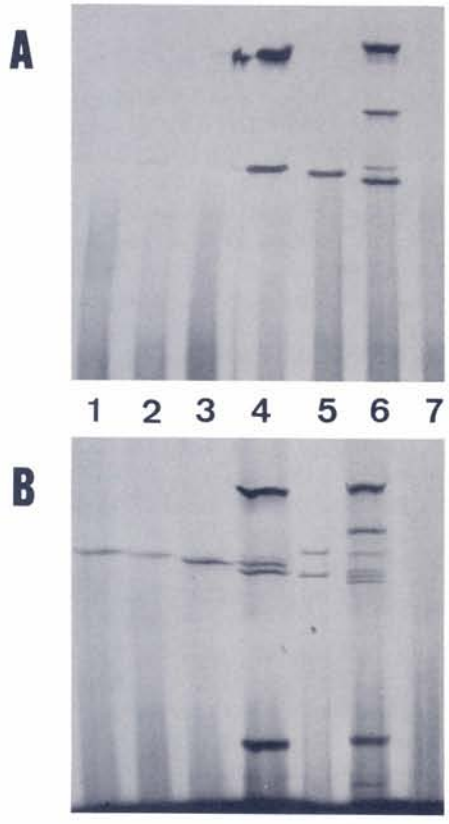

Fig. 1. Sequential-PAGE of nucleic acid preparations from citrons inoculated with field sources and controls, 2 months after inoculation (A), and 3 months after inoculation (B). Positive single viroid controls: 1) CV-IIa, 2) CV-IIb (CCaV), 3) CV-IIc. Field sources: 4) Canoneta sweet orange, 5) Mineola tangelo, 6) Marisol clementine; and 7) uninoculated control.

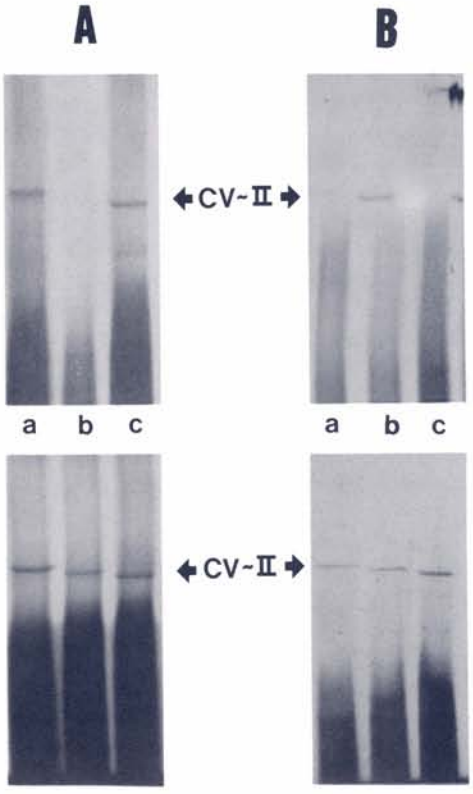

Fig. 2. Sequential-PAGE of nucleic acid preparations from citrons inoculated with positive controls: a) CV-IIa, b) CV-IIb (CCaV), c) CV-IIc. Plants were inoculated in October (A) or in June (B). Plants were extracted 2 months after inoculation (above) and 3 months after inoculation (below).

(Fig. 2). The two plants tested in each assay gave identical results.

Three months after inoculation the level of detection improved considerably (Fig. 1B). The single viroid controls were always detected in the two sets of plants inoculated either in October or June (Fig. 2). The four sets of plants (two sets inoculated in October and two sets inoculated in June) presented identical viroid profiles. The level of detection did not improve when plants were tested later than 3 months after inoculation, or when a second flush of tissue of the same plants was tested again.

Comparison of various electrophoresis systems. As shown above, s-PAGE analysis of nucleic acid preparations extracted from inoculated citrons allowed the detection of CCaV and other citrus viroids 3 months after inoculation (Fig. 3A). The sensitivity for viroid detection was similar when the two-dimensional-PAGE technique described by Schumaker *et al.* (12) was used (Fig. 3B). However, poor results were obtained using the return-PAGE system based on the denaturing properties of heat and low salt conditions (12, 19) (Fig. 3C). As originally described, the technique has been proved adequate to detect potato spindle tuber (PSTV) and crysanthemum stunt viroid (CSV) (Fig. 3C, lanes 8 and 9). It also allows detection of CEV from cit-

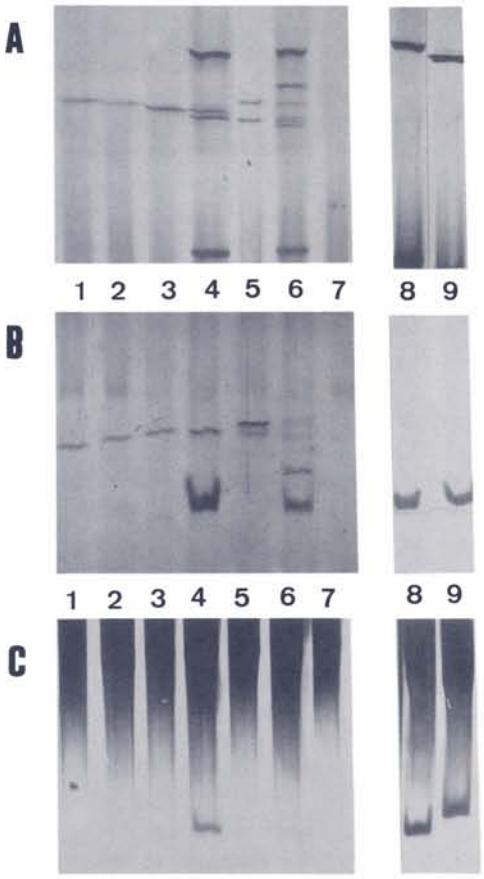

Fig. 3. Nucleic acid preparations from citrons inoculated with field sources and controls, 3 months after inoculation were analyzed using: (A) sequential-PAGE, (B) two dimensional-PAGE, and (C) return-PAGE. Positive single viroid controls: 1) CV-IIa, 2) CV-IIb (CCaV), 3) CV-IIc. Field sources: 4) Canoneta sweet orange, 5) Mineola tangelo, 6) Marisol clementine. Other controls: 7) uninoculated control; and nucleic acid preparations from tomatoes inoculated with 8) PSTV, 9) CSV.

rons infected with field sources containing CEV, provided high titers of the viroid were present in the samples (Fig. 3C, lanes 4 and 6). However the other viroids present in these field isolates were not detected, nor were the single viroids present in the positive controls detected.

Identification of CCaV. As demonstrated above, nucleic acid extraction and s-PAGE allows the detection of citrus viroids from citrons inoculated with field isolates. It was also possible to do a preliminary identification of the viroids into the corresponding citrus viroid groups. Identification of CEV, and CV-I components could be easily done. Note that CEV was detected on Canoneta sweet orange and Marisol clementine (Fig. 1, lanes 4 and 6) and CV-I was detected only on Marisol clementine (Fig. 1, lane 6). Since a continuum of citrus viroids with very close electrophoretic mobilities have been described in the region of migration of CV-II, CV-III and CV-IV viroids (4), the characterization of the viroids into these groups, usually needs further processing. As reported in these proceedings (18), the three viroids of the CV-II group, are associated with two different citrus diseases, exocortis (CV-IIa) and cachexia-xyloporosis (CV-IIb and CV-IIc). Therefore in some instances distinction among the three CV-II components may be desirable. As shown in Fig. 1, the three field isolates tested contained several viroids in the CV-II/CV-III region. As illustrated in Fig. 4, co-electrophoresis of a nucleic acid preparation of Marisol clementine with appropriate markers (a mixture of CV-IIa and CV-IIc), whereas the other three viroid bands

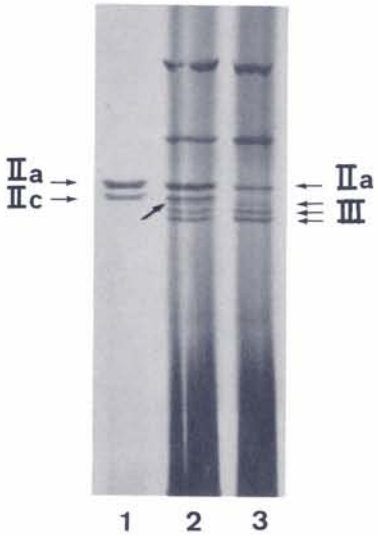

Fig. 4. Sequential-PAGE of nucleic acid preparations of: 1) CV-IIa/CV-IIc, 2) artificial mixture of CV-IIa/CV-IIc and Marisol clementine, 3) Marisol clementine.

had electrophoretic mobilities higher than CV-IIc (note arrow pointing a band with electrophoretic movility only slightly higher than CV-IIc in Fig. 4). Therefore on the basis of this analysis the Marisol sweet orange source was characterized as cachexia-free. The characterization of the three field isolates and the results of biological indexing are summarized in Table 1.

DISCUSSION

Graft inoculation of Arizona Etrog citron 961-S1 followed by nucleic acid extraction and s-PAGE, provides a sensitive and reliable method for detection of CCaV and other citrus viroids. Increasing the sensitivity by running samples equivalent to larger amounts of fresh tissue would require further processing of the nucleic acid preparations. Under our conditions a minimum incubation period of 3 months was required to detect all the viroids present. CCaV and CC-II viroids are usually present in lower titers and seem to need longer incubation periods before they can be detected. Therefore adequate positive controls are recommended in order to perform reliable tests. The differences observed in the assays performed in October and June illustrate possible variations among assays and reinforce the convenience of using more a single positive control.

Under our conditions, two of the three PAGE systems assayed were suitable for detection of citrus viroids. Both systems are equally complex and require the preparation of two separate gels. However, the sharper bands obtained using the s-PAGE system (Fig. 3) are crucial when distinction between CV-II and CV-III viroids is necessary (Fig. 4). The return-PAGE system requires only the preparation of a single gel but it is not usually adequate for detection of citrus viroids.

The detection of CEV by nucleic acid extraction and PAGE has also been shown directly from citrus hosts other than citron (1, 2). Other citrus viroids have also been detected from other hosts (5, 7) but in some instances large amounts of tissue (up to 100 g) were used as starting material. The utilization of citron as an intermediate host in biochemical indexing provides several advantages: a) a natural amplification system for citrus viroids; b) a uniform source of tissue regardless of the host to be indexed; and c) uniform incubation conditions. With the inoculation on citron as an intermediate host followed by nucleic acid extraction and s-PAGE three months later, indexing of cachexia and exocortis can be com-

TABLE 1
BIOLOGICAL AND BIOCHEMICAL INDEXING OF FIELD ISOLATES AND SINGLE VIROID CONTROLS

	Indexing			
	Biological[z]		Biochemical[y]	
Field source	Citron	Parson's	CCaV	Other viroids
Canoneta sweet orange	+ + + +	+ + + +	+	CEV, III
Mineola tangelo	+ +	+/+ + +	+	III
Marisol clementine	+ + + +	−	−	CEV, I, IIa, III
CV-IIa	+	−	−	IIa
CV-IIb	−	+ + + +	+	−
CV-IIc	−	+ + + +	+	−
Uninoculated	−	−	−	−

[z]Results of biological indexing were collected 6 months post-inoculation on citron and 18 months post-inoculation on Parson's Special mandarin. The symptom intensity was rated as: severe (+ + + +); moderate (+ + +); mild (+ +); very mild (+); and no symptoms(−).

[y]Biochemical indexing was run 3 months after inoculation on citron. Detection of CCaV was rated as: positive (+) or negative (−) for the presence of the CCaV.

bined in a single assay for detection of citrus viroids. The method combines the properties of citron as an excellent host for viroids and the sensitivity of s-PAGE and silver staining for their detection. Presently this indexing procedure is routinely used for the Citrus Variety Improvement Program and the Citrus Quarantine Station in Spain.

LITERATURE CITED

1. Baksh, N., R. F. Lee and S. M. Garnsey
   1984. Detection of citrus exocortis viroid by polyacrilamaide gel electrophoresis, p. 343-352. *In* Proc. 9th Conf. IOCV. IOCV, Riverside.
2. Boccardo, G., R. La Rosa and A. Catara
   1984. Detection of citrus exocortis viroid by polyacrilamide gel electrophoresis of nucleic acid from glasshouse citrus, p. 357-361. *In* Proc. 9th Conf. IOCV. IOCV, Riverside.
3. Duran-Vila, N., J. A. Pina, J. F. Ballester, J. Juárez, C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semancik
   1988. The citrus exocortis disease: a complex of viroid RNAs associated with the citrus exocortis syndrome, p. 152-164. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
4. Duran-Vila, N., C. N. Roistacher, R. Rivera-Bustamante, J. S. Semancik
   1988. A definition of citrus viroid groups and their relationship to the exocortis disease. J. Gen. Virol. 69: 3069-3080.
5. Flores, R.
   1988. Detection of citrus exocortis viroid in natural and experimental citrus hosts by biochemical methods. p. 192-196. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
6. Igloi, G. L.
   1983. A silver stain detection of nanogram amounts of tRNA following two-dimensional electrophoresis. Anal. Biochem. 134: 184-188.
7. La Rosa, R., G. Albanesse, A. Azzaro, F. Sesto and F. Domina
   1988. Suitability of nucleic acid analysis to diagnose viroid infections in citrus, p. 188-191. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
8. Pina, J. A., N. Duran-Vila, and L. Navarro
   1991. Interference of citrus viroids with cachexia symptoms on Parson's Special mandarin, p. 206-208. *In* Proc. 11th Conf. IOCV. IOCV, Riverside.
9. Rivera-Bustamante, R., R. Gin, J. S. Semanick
   1986. Enhanced resolution of circular and linear forms of viroid and viroid-like RNA by electrophoresis in a discontinuous-pH system. Anal. Biochem. 156: 91-95.
10. Roistacher, C. N.
    1988. The cachexia and xyloporosis diseases of citrus - a review, p. 116-124. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
11. Roistacher, C. N., R. L. Blue, and E. C. Calavan
    1973. A new test for cachexia. Citrogrpah 58: 261-262.
12. Schumacher, J., J. W. Randles, and D. Riesner
    1983. A two dimensional electrophoretic technique for detection of circular viroids and virusoids. Anal. Biochem. 135: 288-295.
13. Schumacher, J., N. Meyer, D. Riesner, and H. L. Weidemann
    1986. Diagnostic procedure for detection of viroids and viruses with circular RNAs by "Return"-gel electrophoresis. J. Phytopathol. 115: 332-343.
14. Semancik, J. S., T. J. Morris, L. G. Weathers, G. F. Rordorf, D. R. Kearns
    1975. Physical properties of a minimal infectious RNA (viroid) associated with the exocortis disease. Virology 63: 160-167.
15. Semancik, J. S., and K. L. Harper
    1984. Optimal conditions for cell-free synthesis of citrus exocortis viroid and the question of specificity of RNA polymerase activity. Proc. Natl. Acad. Sci. USA 81: 4429-4433.
16. Semancik, J. S., C. N. Roistacher, and N. Duran-Vila
    A new viroid is the casual agent of the citrus cachexia disease, p. 125-135. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
17. Semancik, J. S., C. N. Roistacher, R. Rivera-Bustamante, and N. Duran-Vila
    1988. Citrus cachexia viroid, a new viroid of citrus: relationship to viroids of the exocortis complex. J. Gen. Virol. 69: 3159-3068.
18. Semancik, J. S. and N. Duran-Vila
    1991. The grouping of citrus viroids: additional physical and biological determinants and relationships with diseases of citrus, p. 178-188. *In* Proc. 11th Conf. IOCV. IOCV, Riverside.
19. Singh, R. P., and Boucher
    Electrophoretic separation of a severe from mild isolates of potato spindle tuber viroid. Phytopathology 77: 1588-1591.