Improved Indexing of Citrus Viroids

N. Duran-Vila, J. A. Pina and L. Navarro

ABSTRACT. Inoculation of Arizona 861-S1 Etrog citron followed by nucleic acid analysis by sequential polyacrylamide gel (sPAGE) electrophoresis was evaluated as an alternative for indexing of exocortis, cachexia-xyloporosis and other citrus viroids.

Propagation of citron buds and graft-inoculation with citrus viroids were performed simultaneously on rough lemon rootstock, and plants were incubated at 23-25 C or at 28-32 C. CEVd, CVd-I and CVd-III were detected as early 2-3 months after inoculation, whereas detection of CVd-II and CVd-IV required incubation for at least 6 months. Symptom expression was greatly enhanced when the plants were kept at 28-32 C, and under these conditons, cachexia agents CVd-IIb and CVd-IIc, induced symptoms on Etrog citron.

Nucleic acid analysis of inoculated citrons by sPAGE provides a fast and reliable method for routine indexing of all citrus viroids, and it has relevant advantages over conventional biological indexing.

Index words. exocortis, cachexia, xyloporosis.

Reliable indexing of virus and virus-like agents is required to develop safe quarantine, sanitation and certification programs. The cost of a citrus disease indexing program could be reduced if faster and reliable procedures were available. Unfortunately, detection of many graft-transmissible diseases of citrus still rely on biological indexing which is expensive and time consuming. Particularly, routine indexing of exocortis and cachexia-xyloporosis is still performed by biological indexing on Arizona 861-S1 Etrog citron and Parson's Special mandarin grafted on rough lemon, respectively, which require incubation periods of 6-18 months (3,13,14).

Since viroids were demonstrated to be the causal agents of these two diseases (4,5,17), other detection methods based on common nucleic technologies such as sPAGE, molecular hybridization, and nucleic acid amplification, have been proposed (1,2,7,9,21). In a preliminary study, an alternative procedure to biological indexing of cachexia-xyloporosis was proposed (6). The method combined the excellent properties of citron to obtain detectable viroid titers, and nucleic acid extraction followed by sPAGE and silver staining as the detection tool. Here we present additional data comparing the results of biological indexing and citron inoculation under two sets of incubation temperatures, followed by sPAGE nucleic acid analysis.

MATERIAL AND METHODS

Viroid sources and inoculation. Two sets of plants of Arizona 861-S1 Etrog citron grafted on rough lemon rootstock (15), containing eleven lots of ten plants each, were graft inoculated with: *i*) seven single viroid sources including members of the five different groups of the citrus viroid complex (CEVd, CVd-Ia, CVd-IIa, CVd-IIb, CVd-IIc, CVd-IIId, CVd-IV; *ii*) two combinations of CVd-II viroids (CVd-IIa + CVd-IIb and CVd-IIa + CVd-IIc); and iii) two field sources included as blind controls. These viroid sources were the same used for the characterization of the citrus viroid complex (4,5).

Graft-inoculation of the rough lemon rootstock and propagation of the citron were done simultaneously in October. The plants were kept at 18-25 C until growth of the scion bud was observed in at least half of the plants of each lot. One set of plants was then transferred to a warm greenhouse set at 28-32 C, a temperature considered optimal for biological indexing of viroids, whereas the other set was kept in a cooler greenhouse set at 18-25 C. Symptom development was recorded at monthly intervals.

Viroid detection. Plants from both sets were sampled as soon as enough citron tissue became available (approximately one month after transfer to the final incubation conditions), and at

monthly intervals thereafter. For each inoculation and incubation treatment, samples consisting of approximately 5 g of young leaves and stems were collected from two plants and processed separately, essentially as described by Semancik *et al.* (16). Samples were homogenized in 5 ml extraction medium (0.4 m Tris-HCl, pH 8.9; 1 % SDS, 5mM EDTA, pH 7.0; 4% 2-mercaptoethanol) and 15 ml of water-saturated phenol neutralized at pH 7.0. Total nucleic acids were partitioned in 2 M LiCl, and the soluble fraction was concentrated by ethanol precipitation. The final preparation was resuspended in 300µl TKM buffer (10 mM Tris, 10 mM KCl, 0.1 mM MgCl2, pH 7.4).

Aliquots (equivalent to 300 mg fresh weight tissue) were subjected to 5% sPAGE. The first gel was polymerized in TAE buffer (40 mM Tris, 20 mM sodium acetate·3H20, 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 and 1 cm below was excised and placed on the top of a second gel containing 8 M urea and polymerized with TAE buffer at pH 6.5. The second gel was subjected to a constant current of 16 mA, at room temperature until the tracking dye reached the bottom of the gel (11). The viroids were viewed in the gel after silver staining (8).

RESULTS

Development of symptoms associated with viroid infection during biological indexing. Symptom development was greatly affected by the incubation temperatures (Table 1). All the plants inoculated with CEVd and incubated at 28-32 C showed severe stunting, severe epinasty, and vein and petiole necrosis as soon as the citron scion grew, whereas symptoms in plants kept at 18-25 C were erratic, with variable intensity, and vein and petiole necrosis were rare. Differences in symptom intensity correlated with differences in viroid titers.

Plants inoculated with CVd-Ia developed mild epinasty, and localized midvein necrosis (4,5) in a few leaves (Fig. 1 a). Symptom intensity and the period needed for symptom expression were similar in all inoculated plants regardless of the incubation temperature (Table 1). In most instances the mild epinasty observed initially (Fig. 1a) in plants incubated under warm temperature regimes eventually developed into the variable "syndrome" characterized by very mild leaf epinasty alternating with moderately severe symptoms (Fig. 1b) (18). At the end of the experiment (12 months after inoculation) the presence of pinholing in the cambial face of the bark above the bud union was observed (Fig. 1c). This symptom was specific of CVd-Ia and it was observed even in mixed infections with other viroids.

Plants inoculated with CVd-II viroids only developed symptoms after 6-9 months (Table 1). At 28-32C CVd-II viroids induced necrotic spots in the stem just below the insertion of the petioles (Fig. 2a), and sometimes petiole wrinkling (Fig. 2b). The necrotic spots showed consistently 6 months after inoculation in plants infected with CVd-IIa, whereas plants infected with the two cachexia agents CVd-IIb and CVd-IIc showed only milder symptoms 4 months later. The three CVd-II viroids behaved as true strains in terms of intensity of symptom induced on inoculated citrons and incubation period required for symptom expression, CVd-IIb being the mildest strain and CVd-IIa the most severe. Within the period studied, necrotic spots and petiole wrinkle were rarely observed in plants incubated at 18-25 C (Table 1) and only in plants that had not been pruned.

Plants inoculated with CVd-IIId and incubated at 28-32 C showed mild dwarfing and epinasty resulting from petiole and midvein necrosis, 3 months after inoculation (Fig. 3), whereas plants incubated at 18- 25 C showed symptoms only 6 months after inoculation (Table 1). The symptoms induced by CVd-IV in citron were indistinguishable from those of CVd-III, but longer incubation periods were always necessary (Table 1).

TABLE 1
EFFECT OF INCUBATION TEMPERATURE ON SYMPTOM EXPRESSION OF CITRUS VIROIDS ON ARIZONA 861-S1 ETROG CITRON

Viroid	28-32C				18-25C			
	Incubation period (months)				Incubation period (months)			
	1	3	6	9	1	3	6	9
CEVd	5/5[z]	5/5	5/5	5/5	1/7	4/7	7/7	7/7
CVd-Ia	2/7	7/7	7/7	7/7	0/8	8/8	8/8	8/8
CVd-IIa	0/9	0/9	9/9	9/9	0/10	0/10	0/10	4/10
CVd-IIb	0/10	0/10	2/10	6/10	0/10	0/10	0/10	0/10
CVd-IIc	0/7	0/7	5/7	6/7	0/10	0/10	0/10	1/10
CVd-IIId	0/8	8/8	8/8	8/8	0/9	1/9	9/9	9/9
CVd-IV	0/7	1/7	7/7	7/7	0/10	0/10	1/10	2/10

[z]Figures indicate the number of plants showing symptoms/total number of inoculated plants.

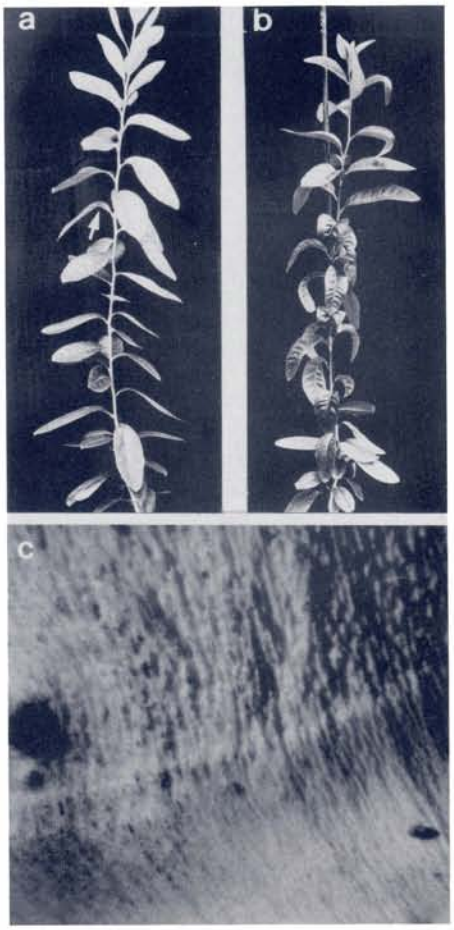

Fig. 1. Symptoms induced by CVd-Ia on Arizona 861-S1 Etrog citron: a) very mild epinasty as a result of localized necrosis observed in some leaves (see arrow pointing at leaf bending resulting from point midvein necrosis); b) "variable" syndrome characterized by mild leaf symptoms alternating with moderate symptoms; c) pinholing observed by lifting of the bark.

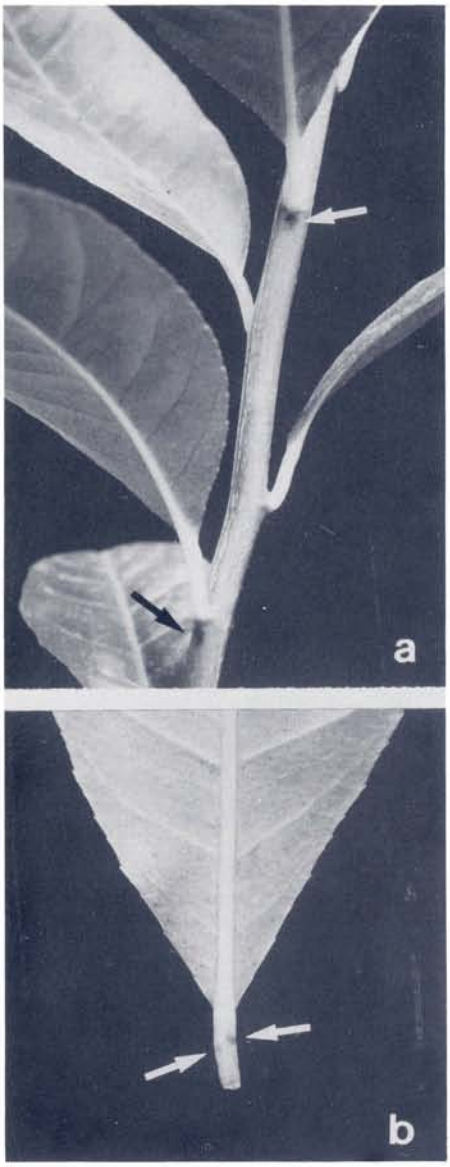

Fig. 2. Symptoms induced by CVd-IIa on Arizona 861-S1 Etrog citron: a) necrotic spots in the stem just below insertion of the petiole (see arrows); b) mild necrosis and wrinkling of the periole.

In summary, upon biological indexing using Etrog citron 861-S1 as indicator, the following was observed:

– Symptom development was affected by the incubation temperature.

– Six months were required for safe indexing of CEVd, CVd-I, CVd-III and CVd-IV, when the inoculated citrons were incubated at 28-32 C, and at least 9 months when incubation was at 18-25 C.

– When the inoculated citrons were not pruned, CVd-II viroids (including the cachexia-xyloporosis agents) were also detected. At least nine months were required for safe detection on plants incubated at 28-32 C. In plants incubated at 18-25 C, longer incubation periods were required (12 months or longer), and the symptoms were erratic.

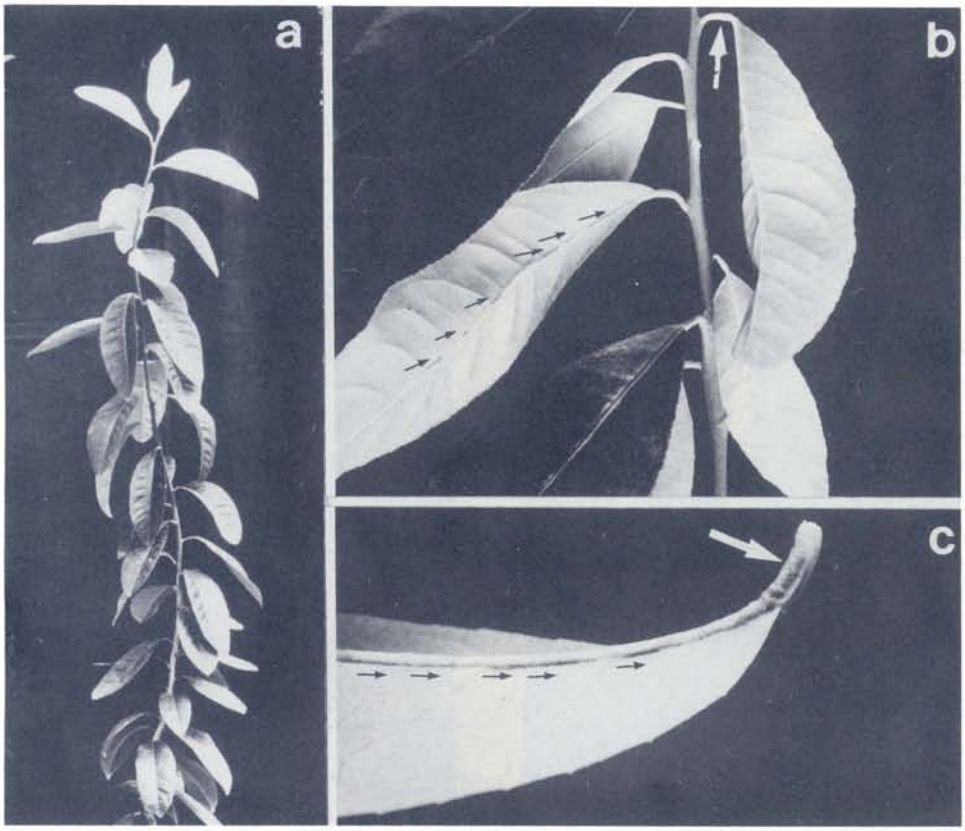

Fig. 3. Symptoms induced by CVd-IIId on Arizona 861-S1 Etrog citron: a) mild dwarfing and epinasty resulting from severe petiole and midvein necrosis; b-c) Midvein necrosis (see small arrows) and bending of the petiole.

Viroid detection by nucleic acid extraction and sPAGE. Regardless of the incubation temperatures, CEVd, CVd-Ia and CVd-IIId were detected as soon as enough tissue was available (after one month incubation period) and CVd-IV one month later (Table 2, Fig. 4 a). However detection of CVd-II viroids was greatly enhanced by incubating the inoculated plants at 28-32 C. At least a 3-month incubation period was necessary for consistent detection of all CVd-II viroids and their mixtures (Fig. 4 b) in plants incubated at 28-32 C whereas four additional months were required to achieve the same results with plants incubated at 18-25 C (Table 2).

The field sources included in this study as blind controls were analyzed after a 3-month incubation period at 28-32 C or a 7-month period at 18-25 C, and at monthly intervals thereafter. The viroid profiles observed in the first analysis (Fig. 4) were the same observed in further analysis.

In summary, on the indexing by nucleic acid extraction and sPAGE of inoculated Etrog citron 861-S1, the following was observed:

– Viroid detection was affected by the incubation temperature.

– When the inoculated citrons were incubated at 28-32 C, 2 months were required for safe indexing of CEVd, CVd-I, CVd-III and CVd-IV, and at least 3 months were required when incubation was at 18-25 C.

– Viroids of the CVd-II group (including the cachexia-xyloporosis agents) were also detected on inoculated citrons (pruned or not). At least 3 months were required for safe detec-

TABLE 2
EFFECT OF INCUBATION TEMPERATURE ON DETECTION OF CITRUS VIROIDS ON ARIZONA 861-S1 ETROG CITRON AND sPAGE ANALYSIS[z]

	28-32C			18-25C				
	Incubation period (months)			Incubation period (months)				
Viroid	1	2	3	1	2	3	5	7
VEVd	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-Ia	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-Ia	− −	+ +	+ +	− −	− −	− −	+ +	+ +
CVd-IIb	− −	+ +	+ +	− −	− −	− −	+ +	+ +
CVd-IIc	− −	− +	+ +	− −	− −	− −	− +	+ +
CVd-IIId	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
CVd-IV	− −	+ +	+ +	− −	+ +	+ +	+ +	+ +

[z]Two plants for each treatment and incubation period were analyzed separately; + + = positive detection in both plants; + − = positive detection in only one of the plants; − − = no detection in either plant.

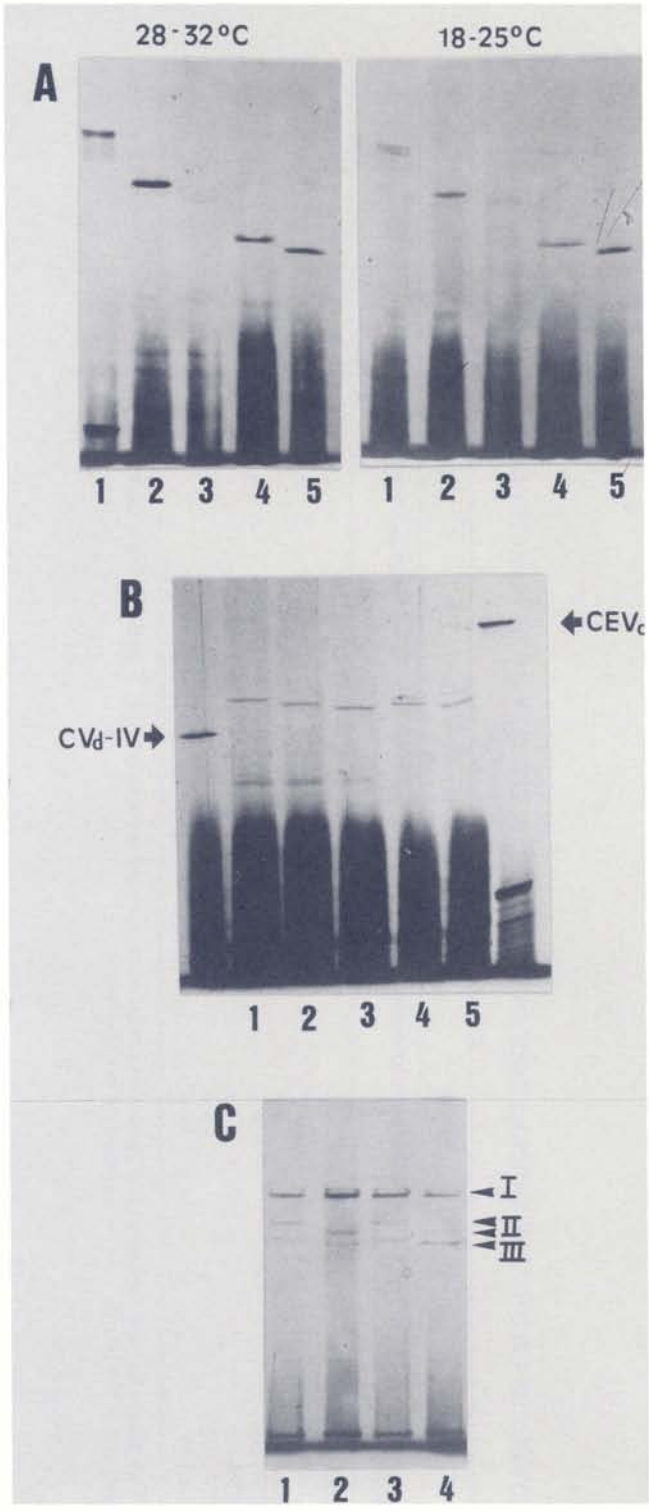

Fig. 4. Detection of citrus viroids by inoculation on Etrog citron and nucleic acid analysis by sPAGE. A) Effect of incubation temperature on detection of CEVd (1), CVd-Ia (2); CVd-IIa (3); CVd-IIId (4); CVd-IV (5). B) Detection of CVd-II viroids (arrows show CEVd and CVd-IV standards): CVd-IIa (1); CVd-IIb (2); CVd-IIc (3); CVd-IIa + CVd-IIb (4); CVd-IIa + CVd-IIc (5). C) Detection of viroids present in two field isolates, inoculated in Etrog citron plants and incubated at 28-32 C (1,2) or at 18-25 C (3,4).

tion on plants incubated at 28-32 C, and 7 months on plants incubated at 18-25 C. Detection was not erratic.

DISCUSSION

Improved detection procedures must be superior to conventional methods in terms of sensitivity, reproducibility, reliability and cost. In an earlier work (6), it was shown that inoculation of citron and analysis of nucleic acid extracts by sPAGE and silver staining was a faster and more reliable procedure for cachexia detection than biological indexing on Parson's Special. The information reported here demonstrates that graft-inoculation on Arizona 861-S1 Etrog citron can be safely used as a single bioassay for detection of all citrus viroids.

Unlike previous reports (4,5,17), the cachexia-xyloprorosis agents CV-IIb and CV-IIc are not symptomless on Etrog citron. They induce mild symptoms in 6-9 months provided that the citron plants are left unpruned. As in the case of the Parson's Special bioassay (13,14), the incubation conditions are critical for symptom expression and the response of the indicator plants is erratic. However, provided that several plants per test and appropriate controls (i.e. CVd-IIb) are used, detection on citron can be achieved with the same sensitivity as the Parson's Special bioassay. The possibility of using a single assay for biological indexing of all citrus viroids would result in a lower number of indicator plants used and in a reduction of the incubation period required. The assay is truly reliable when the plants are left unpruned for 9-12 months and when specificity is not required. In such case the Parson's Special assay still remains the best specific test for the cachexia-xyloporosis agents (12).

The period necessary for a safe diagnosis of citrus viroids can be considerably reduced by analyzing nucleic acid preparations from the inoculated citrons by sPAGE and silver staining (Table 3). In such case the incubation temperature is less critical, the number of indicator plants can be reduced to 1 or 2 per test, and incubation periods of 3 months at 23-32 C or 7 months at 18-25 C are necessary for the detection of all citrus viroids.

Several authors have shown that symptom expression is affected by complex interactions among viroids (10,19,20). In the case of the Parson's Special bioassay this may result in lower sensitivity to detect the cachexia-xyloporosis agents when the related viroid CVd-IIa is present in mixed infections. As illustrated in Fig. 4, the sensitivity of sPAGE analysis is not affected by this kind of biological interaction. In addition, the procedure presented here provides information on the number and kind of viroids present in a given field isolate. This procedure, combining inoculation on citron and sPAGE nucleic acid analysis, was adopted in Spain in 1989 for the routine indexing of plants included in the quar-

TABLE 3
TIME REQUIRED FOR RELIABLE DETECTION OF CITRUS VIROID BY CONVENTIONAL BIOASSAY OR BY sPAGE AFTER INOCULATION ON ARIZONA 861-S1 ETROG CITRON[z]

	28-32 C		18-25 C	
Viroids	Biological indexing	sPAGE on citron	Biological indexing	sPAGE on citron
CEVd	1	1	3-6	1
CVd-I	3	1	3-6	1
CVd-II	6-9	2-3	>9	5-7
CVd-III	3	1	6	1
CVd-IV	6	3	>9	2

[z]Table shows minimum incubation periods in months.

antine, sanitation and certification programs. The standard conditions adopted were: *i*) inoculation of Arizona 861-S1 Etrog citron (2-4 plants per test); *ii*) incubation at 28-32 C without pruning; *iii*) observation of symptoms after 6 months; *iv*) nucleic acid analysis by sPAGE and silver staining. Since then, 435 plants have been tested and the superiority of the new procedure clearly demonstrated.

Since the facilities and general resources available in different countries are variable, no strict guidelines should be dictated to define indexing protocols. However from the results discussed here, the following should be concluded:

a) Incubation of inoculated citron plants at warm temperatures is highly advisable.
b) Minimum incubation periods must be adjusted according to the indexing facilities available. Mild positive controls should always be used.
c) Biological indexing using Arizona 861-S1 Etrog citron allows detection of all citrus viroids, although detection of cachexia-xyloporosis may be as erratic as in the case of the Parson's Special bioassay.
d) Inoculation on citron and sPAGE nucleic acid analysis and silver staining provides a faster, cheaper and more reliable method than conventional biological indexing.
e) Analysis of citrus viroids by inoculation on citron followed by sPAGE and silver staining is not erratic for detection of CVd-II viroids including the cachexia-xyloporosis agents.

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