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# Biochemical Indexing for Citrus Exocortis Viroid

M. R. Gillings, P. Broadbent and B. I. Gollnow

**ABSTRACT.** We used biochemical tests for citrus exocortis viroid (CEV) in conjunction with biological indexing on Etrog citron, to survey mother trees in budwood multiplication blocks for CEV infection. Initial work concentrated on improving the RNA extraction methods and determining the optimum tissue type and time of year for sampling field trees. On a fresh weight basis, young fully expanded leaves in late summer gave the highest yields of CEV. CEV was detected in field and indicator plants using polyacrylamide gel electrophoresis, and by hybridisation of CEV-cDNA probes to RNA dot blots and Northern transfers. These biochemical assays were compared with biological indexing and used to differentiate between infection with CEV and other more benign agents that also cause symptoms on indicator plants. While CEV causes severe symptoms on Etrog citron, other viroidlike RNAs are associated with moderate symptoms. These RNAs are smaller than CEV and show no homology to CEV probes. No RNA agent(s) has, to date, been consistently associated with mild symptoms on Etrog citron.

*Index words.* RNA, PAGE, cDNA probes, dot blots, hybridisation, Etrog citron.

Control of CEV is achieved by use of tolerant rootstocks (7) or elimination of CEV from infected budwood sources by nucellar propagation (27) or shoot tip grafting (15, 18). Maintenance of indexed mother-tree blocks and prevention of root grafting or mechanical transmission by pruning and hedging tools (5, 16) will ensure distribution of CEV-free budwood from these multiplication plantings.

To guarantee that symptomless scion material does not carry CEV, it is essential to have rapid, reliable indexing procedures. Citrus clones carrying CEV were initially detected by the appearance of rootstock bark-scaling and dwarfing symptoms when the material was grafted onto susceptible trifoliate orange or Rangpur lime rootstocks (10, 14). Later, CEV was detected by tissue-graft indexing on citron clones USDA 60-13 (6), Arizona 861 (1) or 861-S-I (17). Symptoms on citron indicators include epinasty, dwarfing and corking of the petioles. Symptoms on citron may appear in 1 to 3 months (6) and may be classified as mild, moderate, or severe (8). But mild and moderate symptoms may be induced by other viroidlike RNA's (8), thus confusing the indexing for CEV.

Biological indexing, as described above, is lengthy, labor intensive, and requires expensive high-temperature/

humidity glasshouses for year-round indexing. However, these procedures can be done by relatively inexperienced staff under supervision.

Polyacrylamide gel electrophoresis (PAGE) has been used to isolate and purify severe strains of CEV (23, 25), and as a means of routine detection of CEV (2, 4). Sequential PAGE analysis of nucleic acids from graft-inoculated citrons with moderate to severe symptoms has revealed the presence of additional viroid RNAs smaller than CEV-RNA. These RNAs are independently transmissible to citron (8). However, agents characterized by mild reactions on citron have been difficult to detect by PAGE (8, 20).

Problems inherent in biological and electrophoretic indexing can be overcome by the use of cDNA probes to CEV (3, 20, 21, 26). Such techniques have so far principally been used as a research tool rather than for large-scale indexing of citrus trees. This paper discusses the use of cDNA-CEV probes for indexing field trees, their limitations when multiple infections with viroidlike RNA species occur and, consequently, the need for combined approaches to indexing, including biological, electrophoretic and cDNA hybridisation assays.

## MATERIALS AND METHODS

**Citrus clones tested.** The severe isolates of CEV were originally obtained from field trees showing bark-scaling symptoms on trifoliolate orange rootstocks. The mild and moderate isolates, as judged by the leaf-curling reactions on citron indicators, were from orchard trees and trees in the NSW Citrus Budwood Multiplication Scheme. They had been indexed as free from psorosis and xyloporosis, but do carry citrus tristeza virus.

**Biological indexing.** Budwood from all sources was grafted onto Arizona 861 citron plants (rooted cuttings) and grown in a heated glasshouse (34 C day/25 C night) under high humidity. Citrons were cut back on several occasions to initiate new growth. All budding and pruning was done with new razor blades or secateurs disinfected in 2% sodium hypochlorite.

**Extraction of nucleic acids.** Procedures for extracting nucleic acids from gram quantities of fresh or frozen tissue are given in Broadbent, *et al.* (5).

**Micro-extraction technique.** To increase the speed with which samples could be processed, a micro-extraction technique was developed. Three to five fresh leaves from field or indicator plants were pressed using an Erich Pollahne sap extractor. Fifteen drops of the exudate were collected into an Eppendorf tube containing 500  $\mu$ l of the following mixture: 3 ml 1X STE (100 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.2), 5 ml phenol (equilibrated with 0.1 M Tris, pH 8.0 and 0.1% w/v 8-hydroxyquinoline), 4 ml extraction medium (100 mM NaCl, 10 mM EDTA, 4% SDS, 100 mM Tris, pH 8.9), 1 ml 20% polyvinylpyrrolidone and 25  $\mu$ l  $\beta$ -mercaptoethanol. AR grade chemicals were used throughout.

The suspension was mixed by shaking and held on ice for 1 h. The phases were separated by a 10-min centrifugation in an Eppendorf 5414 S microfuge, and the aqueous phase

extracted with 400  $\mu$ l chloroform/isoamyl alcohol (24:1 v/v) for 20 min on ice, and separated by centrifugation as above. The nucleic acids were precipitated from the aqueous phase by the addition of 2½ volumes of cold (-20 C) absolute ethanol. After 1 h at -20 C the nucleic acids were pelleted by centrifugation, washed in cold 70% ethanol, 100 mM Na acetate, briefly air dried and resuspended overnight at 4 C in 100  $\mu$ l of 1X STE. The nucleic acid solution was clarified by centrifugation and transferred to a new tube.

**Polyacrylamide gel electrophoresis.** Total nucleic acids from field or indicator plants were analysed on polyacrylamide gels (13). One tenth (10  $\mu$ l from 100  $\mu$ l) of the nucleic acids from the micro-extraction technique was loaded onto 80 x 80 x 2 mm 5% polyacrylamide gels poured with TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.3). Gels were run in the same buffer at 8V/cm until the bromophenol blue marker had migrated out of the gel. Gels were stained for 1 h with 0.5  $\mu$ g/ml ethidium bromide in TBE buffer, and briefly destained by rinsing in TBE.

Where appropriate, the section of the gel containing the CEV and 7S RNA bands was cut out and the nucleic acids run into an 80 x 160 x 2mm 5% polyacrylamide gel poured in 0.6 x TBE pH 7.2 and containing 8M urea (8). Gels were run in TBE, pH 8.3, stained with ethidium bromide and then with silver using the method of Igloi (12).

**Dot blotting.** Nucleic acid samples were prepared for dot blotting by a modification of White and Bancroft (28). Three to five microliters of sample (of 100  $\mu$ l) were added to 195  $\mu$ l of a denaturing solution consisting of 10X SSC [1X SSC = 0.15 M NaCl, 0.015 M Na<sub>3</sub> citrate], 10 mM sodium phosphate, pH 7.0 and 8% formaldehyde. One in five serial dilutions were prepared by transferring 40  $\mu$ l of the nucleic acid mix to 160  $\mu$ l of the

denaturing solution. All manipulations were done in 96-well microtiter trays. To denature the nucleic acids, the microtiter tray was floated in a 55 C water bath for 30 min and then chilled on ice.

One hundred microliters of the denatured nucleic acid samples were loaded onto nitrocellulose (S&S BA85 pretreated in distilled water, then 20X SSC) using a BioRad "Bio Dot" vacuum manifold. Wells were rinsed with 0.5 ml 20X SSC, and the filter air dried overnight. The filter was baked at 80 C for 2 h to fix the nucleic acids, and was then washed in distilled water to remove excess salt. Filters were air dried and stored in sealed plastic bags until use.

**Hybridizations.** Hybridizations were carried out by a modification of standard procedures (13). Filters were placed in plastic bags, wet with distilled water and drained. Prehybridization solution was then added to 0.2 ml/cm<sup>2</sup> and the sealed bags incubated in a shaking water bath at 42 C for 3 h. The prehybridization solution contained 50% formamide (deionized), 4X SSC, 5X Denhardt's solution (1X DS = 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) BSA), 0.05 M sodium phosphate, pH 6.5, 1 mg/ml sheared denatured calf thymus DNA, 5% sodium dextran sulphate and 0.25% SDS. The SSC, DS, and PO<sub>4</sub> buffer were filtered through 0.45 µm nitrocellulose before use.

At the end of the prehybridization, the bags were drained, and 50 µl/cm<sup>2</sup> of hybridization solution added. The hybridization solution was 50% formamide, 4X SSC, 1X DS, 12 mM Na PO<sub>4</sub> pH 6.5, 100 µ/ml denatured calf thymus DNA, 10% sodium dextran sulphate, 0.25% SDS containing 5 x 10<sup>5</sup> cpm/ml of a <sup>32</sup>P-labeled CEV probe.

CEV probes were made by Biotechnology Research Enterprises, S.A., Pty. Ltd., and were negative sense single-stranded cDNAs prepared from full length CEV-A cDNA in an M13 mp93 vector. The specific

activity of the probes was about 1.5 x 10<sup>6</sup> cpm/µg.

Hybridisation was at 42 C in a shaking water bath for 16 h. At the end of the hybridization, the bags were drained and the filters washed twice at room temperature in 100 ml of 2X SSC, 0.5% SDS. Filters were then washed for 1 h at 42 C, and for 30 min at 55 to 60 C in the same solution. Filters were blotted dry and used to expose X-ray film with intensifying screens at -80 C. Exposures ranged between 1 and 5 days.

## RESULTS AND DISCUSSION

Rapid and reliable detection of virus and viroid diseases is essential to any budwood multiplication program. Not only can failure to detect disease result in the distribution of large amounts of infected propagating material and substantial losses to the orchardists, but it may involve the agency responsible for the indexing in costly litigations.

CEV can now be detected by a number of methods, both biological and biochemical. The usefulness of these methods depends on the number of trees to be indexed, the facilities available and the expertise of staff.

The experiments reported here are directed towards establishing routine biochemical assays for CEV, and to establish some of the parameters important for accurate diagnosis. These biochemical assays are dependent on detection of CEV-RNA.

**Extraction of nucleic acids.** Several extraction procedures were examined. We chose that of Flores, *et al.* (9) on the basis of its speed and simplicity. Minor modifications to the protocol (5) enabled the extraction of good yields of high molecular weight RNA from citrus. The method can be used successfully on fresh or frozen tissue, and on tissue stored at 4 C.

This method was modified for use with an electric leaf press (see Methods), with the consequent ad-

TABLE 1  
COMPARISON OF RESULTS OF CITRUS EXOCORTIS VIROID (CEV) INDEXING USING VISUAL ASSESSMENT OF BARK SCALING OF TRIFOLIATE ORANGE ROOTSTOCK, BIOLOGICAL INDEXING ON ETROG CITRON AND cDNA PROBE TO CEV

Clone or variety	Row/tree	Rootstock <sup>z</sup>	Rootstock Symptoms (Bark scaling)	Indexing	
				Etrog citron	cDNA probe <sup>x</sup>
Accession No. Lambert Eureka 2013	R <sub>22</sub> T <sub>97</sub>	R.L.	N/A <sup>y</sup>	+	-RB
Villa Franca 3658	R <sub>20</sub> T <sub>100</sub> T <sub>102</sub> T <sub>103</sub> T <sub>104</sub> T <sub>105</sub> T <sub>109</sub>	tri tri tri tri tri tri	+ + + + ? ?	+ + + + - -	+RB +TB +RB N.D. - -
Prior Lisbon 3924	R <sub>19</sub> T <sub>71</sub> T <sub>72</sub> T <sub>74</sub> T <sub>78</sub> T <sub>86</sub> T <sub>87</sub>	tri tri tri tri tri tri	+ + + - + +	- - - + + +	- - - +RB +TB +RB
Taylor Eureka 3402	R <sub>20</sub> T <sub>72</sub> R <sub>20</sub> T <sub>73</sub> R <sub>19</sub> T <sub>96</sub> R <sub>19</sub> T <sub>98</sub> R <sub>19</sub> T <sub>99</sub>	R.L. R.L. Cleopatra Cleopatra Cleopatra	N/A N/A N/A N/A N/A	+ + + + +	N.D. N.D. +L, +RB +L N.D.

<sup>z</sup>R.L. = rough lemon; tri = trifoliolate orange.

<sup>y</sup>N/A = not applicable as rootstock was not trifoliolate orange.

<sup>x</sup>RB = bark from rootstock; TB = bark from twigs; L = Etrog citron leaves.

N.D. = not done.

vantages that smaller amounts of sample and chemicals were required, and more samples could be processed. Extraction using the leaf press is most efficient with indicator plants, and can be used on leaves from field trees, provided these are turgid.

**PAGE analysis.** Nucleic acids extracted from field and indicator plants were analysed on slab polyacrylamide gels. CEV can be detected in symptomatic citron indicators as a single band running above the 7S RNA. (fig 1; tracks b, i and j). In our hands LiCl partitioning (22) does not measurably improve detection of CEV by PAGE (see fig. 1).

When RNA from infected citrons is inoculated onto chrysanthemum, the titres of CEV are much higher, and as a consequence CEV is more

easily detected (fig. 1; track k). RNA from field trees known to carry CEV did not exhibit a CEV band on gels stained with ethidium bromide (fig. 1; track l), even though these samples gave approximately the same response to CEV probes as did infected citrons. CEV in established field infections may occur as multimeric molecules, not easily detected by PAGE. Northern transfer (RNA) analysis showed that CEV is present as multimers (up to 5-mer) in some infected plants (evidence not presented). This observation cautions against the routine use of PAGE for diagnosis of CEV in tissue from field trees.

The use of PAGE for indexing very large numbers of field samples may be inappropriate due to the cost

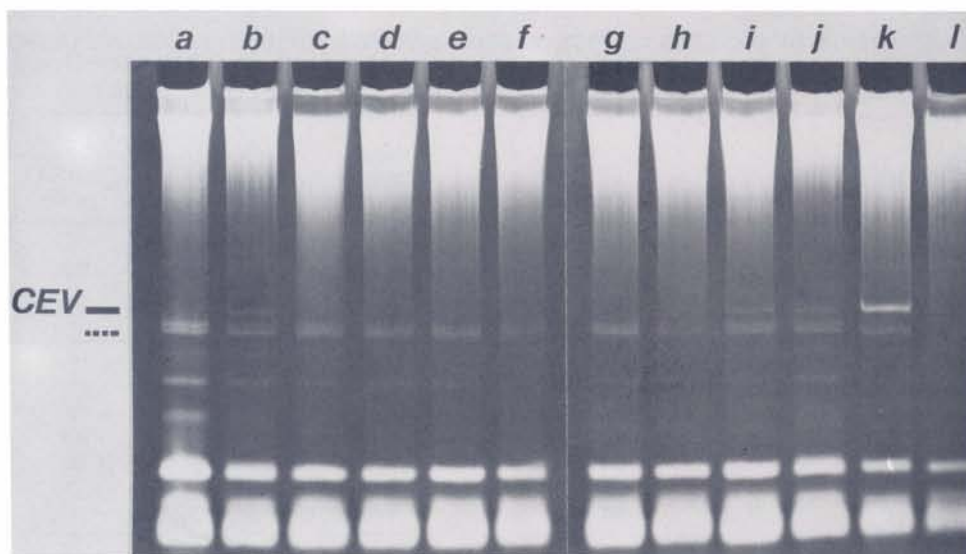


Fig. 1. Analysis of RNA on 5% non-denaturing polyacrylamide gel. Stain: Ethidium bromide. All samples are the nucleic acids from 0.25 g of leaf tissue. Tracks are as follows: a) Uninoculated citron; b) Citron bud inoculated (/) with Taylor Eureka lemon (3402) R19 T96 (CEV +); c) Citron / Koster Ellendale Tangor 'Parr'; d) Citron / Koster Ellendale 10<sup>0</sup>; e) Citron / Koster Ellendale 10<sup>0</sup>; f) Citron / Koster Ellendale R6 T5; g) Citron / Koster Ellendale R6 T6; h) Citron / Hamlin Orange (3446); i) Citron / Frost Eureka lemon (4334) (CEV +); j) Citron / Taylor Eureka lemon (3402) R19T96 (CEV +); k) Chrysanthemum / 033 (CEV +); l) Eureka lemon (field sample CEV +). Tracks c-h are LiCl soluble fractions, all others are total nucleic acids. Citrons b, i, and j showed severe leaf curling, c-g moderate symptoms and h, mild symptoms.

of biochemicals, the need for purification and electrophoresis equipment and the requirement for technical expertise (2).

**Hybridisation assay.** Total nucleic acids from field or indicator plants were spotted onto nitrocellulose and assayed for CEV-RNA using hybridization with a negative sense CEV-cDNA probe. Field plants indexed in this way are shown in fig. 3 of Broadbent, *et al.* (5). Hybridisation assays have been used to determine the optimum tissue type and time of year for CEV detection (dot blots not presented). On a fresh weight basis, recently matured leaves of field trees or citron indicator plants give the highest yield of CEV. Leaves are certainly the most convenient tissue to work with, as they can be used with the leaf press and their harvest does not compromise field trees as does the harvest of rootstock or scion bark. However, the leaves must be fully expanded, as succulent leaves early in the growth flush have lower

titres of CEV than leaves farther down the branch.

The optimum time of year for sampling field trees is more equivocal. Positive responses to CEV probes were obtained from all tissues (leaves, twig bark, scion bark, rootstock bark and roots) taken at all times of year (spring, summer, autumn, winter). Individual trees do show some variation, but in general, the highest titres of CEV were found in summer.

That the highest titres of CEV were found in newly expanded leaves in spring or summer, and that biological indexing is enhanced by conditions favouring rapid growth, suggest that titres of CEV are dependent on the metabolic activity of the infected tissue. Baksh, *et al.* (2) found more viroid in young than in older hardened tissue, and Semancik, *et al.* (24) observed that viroid synthesis is favored by cell division or occurs in intimate association with mitotic activity.

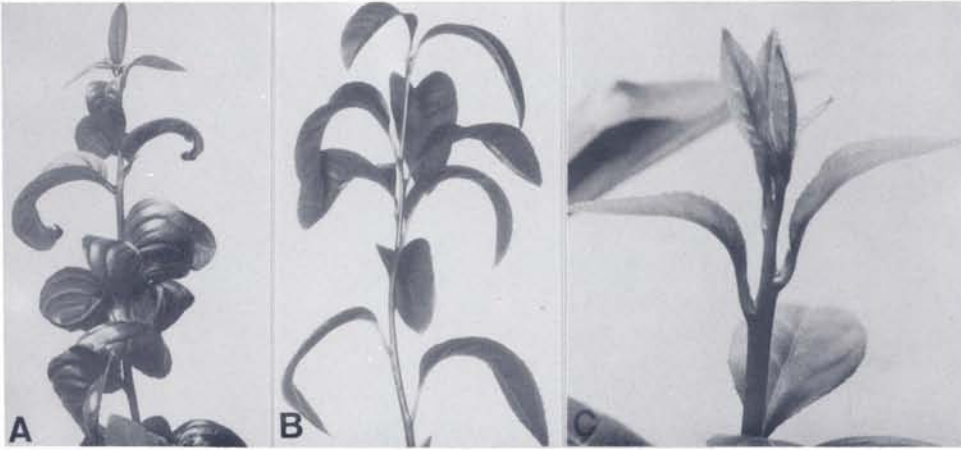


Fig. 2. Biological indexing on citron 861 showing (a) severe symptoms produced by citrus exocortis viroid. CEV isolate (Qld. Villa Franca 4339), (b) moderate symptoms produced by inoculation with Koster Ellendale tangor (CVaV suspected) and (c) mild symptoms produced by dwarfing budline 3532 (causal agent undetected).

**Comparison of biological and biochemical indexing.** Under the conditions used for biological indexing, citron indicators inoculated with CEV-infected buds showed severe and persistent symptoms, including severe epinasty, stunting and, often, death of the terminal shoot (fig. 2a). Symptom onset occurred in 4-8 weeks, and RNA from such plants always showed a CEV band by PAGE (fig. 1), and a positive response to the dot-blot hybridization assay (fig. 3; A1-2, D4).

Other citron indicators showed more moderate and transient symptoms. These plants showed leaf kinking and epinasty, but the symptoms took longer to appear and often affected only a few leaves (fig. 2b). PAGE analysis of RNA from citrons with such moderate symptoms did not show a characteristic CEV band (fig. 1; tracks c-h). However, the citrons inoculated with buds from five independent sources of one budline (Koster Ellendale tangor 4341, including healthy Herps Ellendale tangor trees inoculated with Koster buds), did have a faint ethidium bromide-staining band just below the 7S RNA (fig. 1; tracks c-g dotted line). This band was not present in citron controls, in citrons inoculated with CEV-

infected buds, or in citrons bud-inoculated with other lines inducing mild to moderate symptoms.

To confirm the existence of this faint band, a segment of an ethidium bromide-stained gel was excised and layered onto a 5% polyacrylamide gel containing 8M urea (8). The excised fragments were run into the denaturing gel and stained with silver. Under these conditions, circular viroid RNAs migrate slowly due to their secondary structure. Citron indicators with severe symptoms have a strong CEV band (fig. 3; tracks a and b). CEV can also be detected in infected field trees as a faint band (fig. 3; track c) using the more sensitive silver staining technique (fig 1; track l). Citrons bud-inoculated with independent sources of Koster Ellendale tangor, and showing moderate symptoms, all carry a circular RNA species smaller than CEV (fig. 3; tracks f-h). Since this RNA is not found in control or CEV-infected citrons, it is probably an infectious viroid or viroidlike agent.

To test if this agent was related to, or a deletion mutant of CEV, nucleic acids from citron indicators budded with Koster Ellendale tangor were assayed for CEV by dot-blot hybridization. Citrons inoculated with

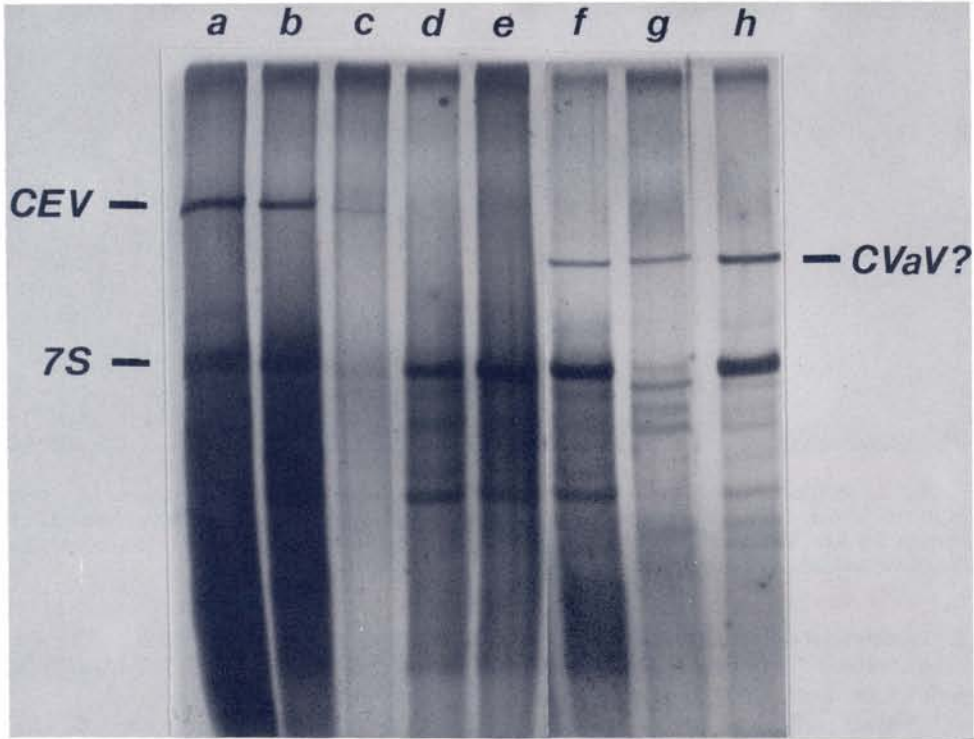


Fig. 3. Analysis of RNA from citron indicators on 5% denaturing polyacrylamide gels. Samples were separated on 5% non-denaturing gels, and the 7S RNA/CEV region excised and layered onto a 5% polyacrylamide gel poured in 0.6X Tris-borate-EDTA (TBE), pH 7.2 and containing 8M urea. After electrophoresis, the gels were stained with silver. All samples are from 0.25 g of leaf tissue.

Tracks are as follows: (a) Citron bud inoculated (/) with Queensland Villa Franca 4339 (CEV +); (b) Citron / Bellamy Navel 3536 (R3T13 dwarfing budline, CEV +); (c) Eureka Lemon 033 (R5T10 field tree, CEV +); (d) Citron / Bellamy Navel 2006 (R1T7 dwarfing budline control); (e) uninoculated citron; (f) citron / Koster Ellendale Tangor R6T6; (g) citron / Koster Ellendale Tangor 'Parr', (h) citron / Koster Ellendale Tangor '10'.

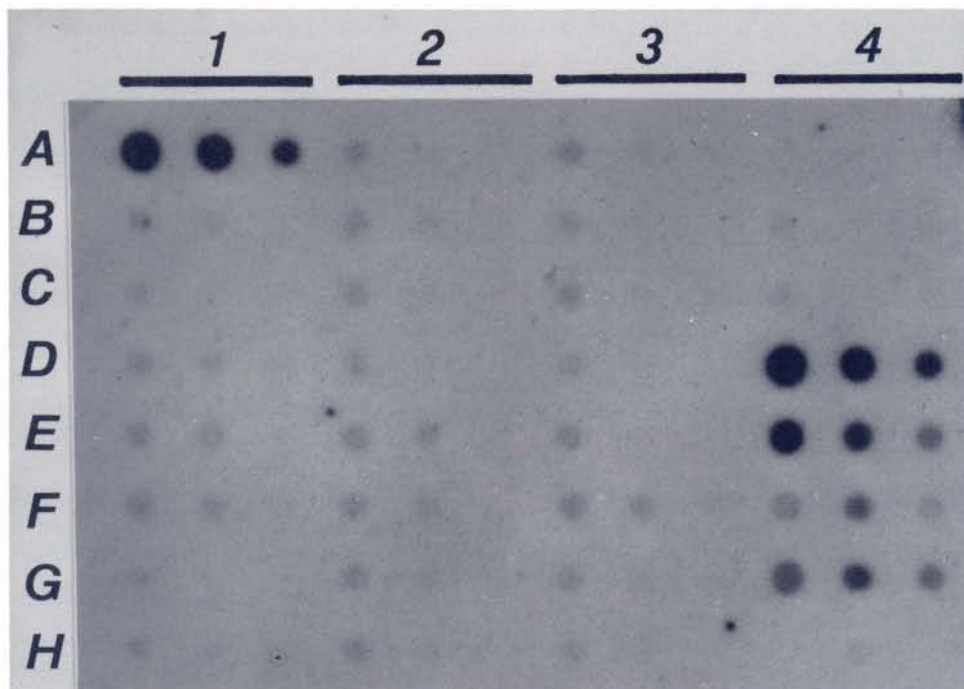
other budlines that also induced mild CEV-like symptoms were similarly treated, as were known CEV-infected citrons and RNA samples containing two viroids with considerable (60-73%) sequence homology to CEV: potato spindle tuber viroid (PSTV) and chrysanthemum stunt viroid (CSV) (11).

The results of this hybridisation are shown in fig. 4. Citrons inoculated with known CEV sources showed a strong response to CEV probes (fig. 4; A1-2, D4, and E4). The two related viroids PSTV and CSV also showed a response to the CEV probe under the hybridisation conditions used (fig. 4; F4 and G4). All other samples taken from citrons with mild to moderate

CEV-like symptoms showed no greater response to the CEV probe than did uninoculated citron controls (fig. 4; A3-4). This indicates that the RNA species in the Koster Ellendale tangor lines is unrelated to CEV. This agent may be the citron variable viroid (CVaV) reported by Schlemmer, *et al.* (19), as its symptomatology and molecular weight (about 20% smaller than CEV) correspond with those reported for CVaV. We have not confirmed the identity of this agent by hybridisation with CVaV-cDNA, nor have we been successful in inoculating gynura or chrysanthemum indicators with this agent.

Several other budwood sources including dwarfing, nonscaling budlines





**Fig. 4. Hybridization assay for CEV in citron indicators showing moderate or mild symptoms. All samples were independent extractions and were spotted at 3 fivefold dilutions.**

**Controls:** A1-2—Citron bud inoculated with Taylor Eureka lemon (3402) R19 T96 (CEV+); A3-4—Citron stab inoculated with healthy citron RNA; D4—Chrysanthemum stab inoculated with the RNA in A1-A2; E4—Chrysanthemum stab inoculated with CEV isolate AS3531 (21); F4—Chrysanthemum stab inoculated with CSV; G4—Tomato plant stab inoculated with PSTV isolate F9.

**Experimental:** B1 and C1—citron inoculated with Koster Ellendale tangor 'Parr'; D1 and E1—citron inoculated with Koster Ellendale tangor 10<sup>5</sup>; F1, G1 and H1—citron inoculated with Koster Ellendale tangor 10<sup>6</sup>; B2, C2 and D2—citron inoculated with Koster Ellendale tangor, R6T5; E2, F2, G2 and H2—citron inoculated with Koster Ellendale tangor, R6T6; B3 and C3—citron inoculated with Marsh grapefruit (dwarfing budline 3539); D3 and E3—citron inoculated with Marsh grapefruit ('Perricoota' 4105); F3, G3 and H3—citron inoculated with Hamlin orange 3446; B4—citron inoculated with Clementine mandarin 3922; C4—citron inoculated with Houghton navel 3779.

(5) gave mild, transient symptoms on citron indicators (fig. 2c). We have been unable to detect CEV homology or consistently detect specific RNA bands by PAGE in RNA from citrons inoculated with buds from Hamlin orange 3446, Marsh grapefruit ('Perricoota' 4105), Clementine mandarin (3922) or Houghton navel (3779) (fig. 4.)

In conclusion, biological indexing on citron under favourable conditions enables the detection of not only CEV, but viroid-like RNA's such as RNA I and III (8) and citron variable viroid (19), all of which cause mild to moderate symptoms on citron indi-

cators. The verification of the causal agent(s) of mild leaf curling in citrons requires a multifaceted approach, utilising transmission to other indicator plants (gynura, tomato, chrysanthemum, etc.), PAGE analysis of nucleic acids and dot-blot hybridisation assays. It should be noted that CEV can be detected in dilutions of 100-200 times that required for PAGE when hybridisation assays are used (fig. 4 vs. fig. 1). Where suspected viroid-like agents are not homologous to CEV or to other viroid cDNA probes, PAGE and transmission experiments are the best alternative to define the causal agent(s).

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