Detection of Citrus Exocortis Viroid by Polyacrylamide Gel Electrophoresis of Nucleic Acid Extracts from Glasshouse Citrus[1]

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ABSTRACT. A PAGE technique has been developed for possible diagnostic detection of citrus exocortis viroid (CEV) in relatively crude nucleic acid extracts of citrus roots and leaves from the glasshouse or the field. It combines the sensitivity of silver staining and the selectivity of bidirectional electrophoresis. In preliminary experiments CEV was detected from leaf samples of citron and mandarin, and root samples of volkamer lemon collected in the glasshouse and from sweet orange leaves collected in the field. These results suggest that implementation and assessment of the technique could make possible reliable diagnosis of CEV from field material.
Index words. Exocortis, viroid, two dimensional polyacrylamide gel electrophoresis.

Citrus exocortis viroid (CEV) is usually detected by inoculation of suspected tissues to Etrog citron budlings or seedlings (4). A sensitive clone of citron (861-S-1) reacts well to mild isolates (13), under proper conditions of temperature and plant growth (12).

While this is a great advance over the 5 to 8 year test on trifoliate orange, this method of indexing still has some drawbacks: (i) test plants showing no reaction must be kept under observation for up to one year; (ii) conditions appropriate for maximum CEV symptom expression are not always available, (iii) the high temperatures required are very costly to maintain in winter; and (iv) contamination of mother and/or test plants may occur during the long test period.

For the above reasons, attempts hav been made to develop histochemical (6) methods biochemical procedures such as thin layer chromatography (5, 8) and a combination of column chromatempts have been made to develop tography and polyacrylamide gel electrophoresis (PAGE) (1, 2) for diagnosis. In this paper, we describe a PAGE technique on relatively crude nucleic acid extracts of citrus trees from the glasshouse or the field for possible diagnosis of CEV.

MATERIALS AND METHODS

Root and leaf samples of field-grown Clementine trees grafted on trifoliate orange and of Tarocco sweet orange grafted on sour orange which previously tested positive for CEV infections on Etrog citron, were collected at various times from spring to summer and kept at –20°C. CEV slash-inoculated Etrog citron and bud-grafted Parsons Special mandarin (both on volkamer lemon) were kept in the glasshouse at a temperature of about 26 C (22-32 C). Young symptomatic and fully expanded asymptomatic leaves of citron and mandarin, and roots of Volkamer lemon grafted to citron were used for extractions.

Two to 20 g samples, either freshly collected from the glasshouse or stored at –20 C, were placed in liquid nitrogen and ground to a powder with a mortar and pestle in the presence of either 2 vol (v:w) of 0.05 M tris (hydroxymethyl) aminomethane (Tris), pH 8.5, containing 0.25 M NaCl, 0.25% (w:v) sodium dodecyl sulphate (SDS), 12.5 mM magnesium

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acetate, 2.5% (w:v) polyvinylpyrrolidone (M.W. 40,000) (PVP), 1% (w:v) sodium azide, 0.5% (w:v) sodium diethyldithiocarbamate and 1% (v:v) 2-mercaptoethanol and further processed as described by Palukaitis and Symons (10) for the extraction of CEV from *Gynura aurantiaca* DC (method A) or in the presence of 3 vol (v:w) of an extraction solution consisting of 0.2 M tris, 0.1 M NaCl, 10 mM ethylenediaminotetraacetate (disodium salt) (EDTA), 2% (v:w) PVP, pH 8.5-9.5 (method B). In the latter case, the slurry was homogenized with 1 vol of water-saturated phenol containing 0.1% (w:v) 8-hydroxyquinoline and 1 vol of a 20:1 mixture of chloroform: isoamyl alcohol. After low-speed centrifugation, the nucleic acids were recovered from the aqueous phase by addition of 2 vol of cold ethanol and low-speed centrifugation after 1 hour at –20 C. After fractionation with 2 M LiCl, the Li-soluble fractions were recovered and electrophoresed in 5% polyacrylamide gel slabs buffered in 90 mM Tris, 90 mM borate, 3 mM EDTA, pH 8.3. After completion of the run, one of two sister gels was briefly surface-stained with O-toluidine blue and the other used for the casting of the second direction polyacrylamide gel containing 8 M urea as described by Schumacher *et al.* (14). Second direction gels were stained with silver as for proteins (7). Chrysanthemum stunt (CSV) (9) and coconut cadang-cadang (CCCV) (11, 3) viroids were co-electrophoresed as markers.

RESULTS

In preliminary experiments, method A combined with the use of 5% non-denaturing gels (3) gave inconsistent results with field or glasshouse sampled materials.

By adopting method B for extraction and the PAGE technique of Schumacher *et al.* (14) we obtained consistent results from glasshouse-grown citron and mandarin. A typical first direction (non-denaturing) gel, is shown in fig. 1. The visible bands, in the area where viroids are expected to migrate are those of CSV and CCCV only, the high background, possibly due to nucleic acid degradation, presence of polysaccharides and/or polyphenols, impairing the visualization of CEV bands, if present. As described by Schumacher *et al.* (14), the zone of the sister gel, between the two dashed lines superimposed in fig. 1, was cut out and placed across the bottom of the gel chamber, this being filled with the denaturing gel containing 8 M urea. The electrophoresis was then done in a second direction with inverted polarity, as specified in the legend to fig. 2. Because of the conformational changes incited by the denaturation (14), the electrophor-

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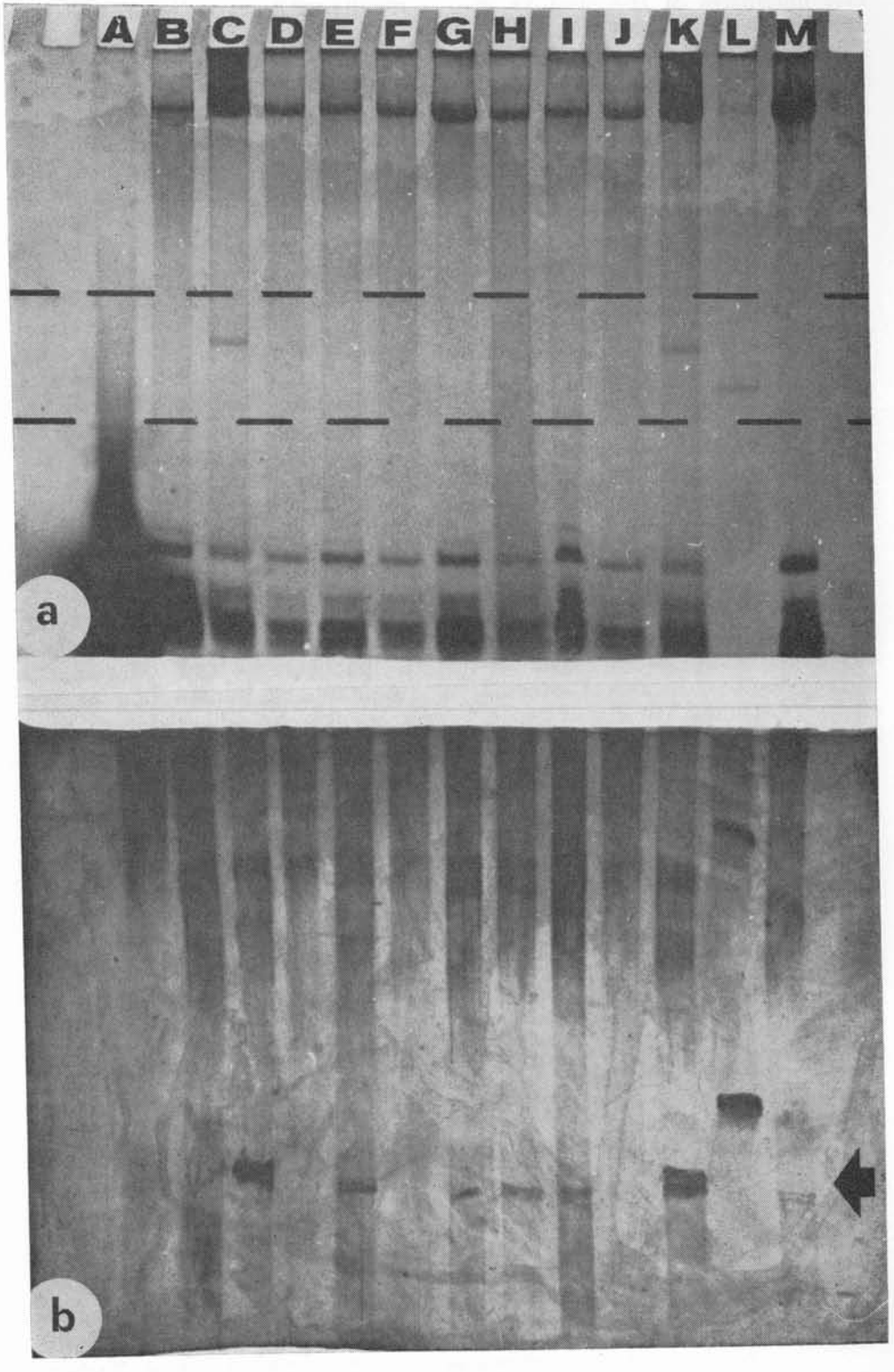
Fig. 1a. Single direction electrophoresis in non-denaturing 5% polyacrylamide gel slab, loaded with nucleic acids extracts of: A, field-collected clementine tangerine leaf tissue; B, healthy Chrysanthemum morifolium**; C and K, chrysanthemum stunt viroid (CSV)-infected** C. morifolium**; D, F and J, healthy citron leaf tissue, from the glasshouse, E, leaves of citrus exocortis viroid (CEV)-inoculated citron, with symptoms; G, same as E, but symptomless; H, roots of volkamer lemon rootstock of inoculated citron; I, CEV-infected symptomless 'Parson Special' mandarin leaves from the glasshouse; L, coconut cadang-cadang viroid (CCCV)-infected coconut; M, young leaves of a field-grown CEV-infected Tarocco sweet orange. Electrophoresis was at 10 mA for 30 minutes followed by 2 hours at 35 mA. Staining: O-toluidine blue. The zone of a sister gel between the two dashed lines was used to cast the second direction gel shown in Fig. 1b.**

Fig. 1b. Second direction PAGE of samples from Fig. 1a. Second direction electrophoresis was done vertically upwards under denaturing conditions (8 M urea, 225 V, 50C), and gells were stained with silver. Arrow indicates viroid bands.


A B C D E F G H I J K L M
a
b

etic mobilities of the circular viroid molecules are considerably slowed-down compared to linear molecules of similar mass, which under non-denaturing conditions would co-migrate (as in fig. 1; first direction gel). This allowed the detection of CEV from glasshouse samples of young leaves of citron, with or without symptoms, roots of volkamer lemon used as rootstock and leaves of Parsons Special mandarin. From samples collected in the field only those of tarocco sweet orange, taken from a heavily pruned tree, gave positive results, while tissues from trifoliate orange and clementine did not give consistent results.

DISCUSSION

Combining sensitive silver staining and selective electrophoretic conditions (7, 14) we have been able to detect CEV in relatively crude nucleic acid extracts from four species of infected citrus trees using 2 to 20 g of tissue. So far we have obtained consistent results with citron tissue, with and without symptoms, symptomless Parsons special mandarin tissue and roots of volkamer lemon from the glasshouse, and Tarocco sweet orange tissues from the field. Erratic results were obtained with symptomless leaves of Clementine (and roots of trifoliate orange) from the field. However, use of the technique described here and of those reported earlier for CEV (1) together with methods for large-scale purification of viroids (7), may offer a reliable diagnostic technique for field material infected with mild or severe CEV strains. This would obviate the need for glasshouse grafting and/or inoculation.

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LITERATURE CITED

1. BAKSH, N., R. F. LEE, and S. M. GARNSEY
   1982. Detection of citrus exocortis viroid from citron by polyacrylamide gel electrophoresis. Phytopathology 72: 354. (Abstr.).
2. BAKSH, N., R. F. LEE, and S. M. GARNSEY
   1983. Detection of citrus exocortis viroid by polyacrylamide gel electrophoresis. p. 343-352. *In* Proc. 9th Conf. IOCV, IOCV. Riverside.
3. BOCCARDO, G., R. G. BEAVER, J. W. RANDLES, J. S. IMPERIAL
   1981. Tinangaja and bristle top, coconut diseases of uncertain etiology in Guam, and their relationship to cadang, cadang disease of coconut in the Philippines. Phytopathology 71: 1104-1107.
4. CALAVAN, E. C., E. F. FROLICH, J. B. CARPENTER, C. N. ROISTACHER, and D. W. CHRISTIANSEN
   1965. Rapid detection of exocortis in citrus. California Agriculture 19: 8-10.
5. CATARA, A., and G. CARTIA
   1971. Possibilità d'impiego di un metodo cromatografico per la diagnosi delle infezioni di "exocortite" degli agrumi in materiale siciliano. Riv. Pat. Veg., S. IV, 7: 173-180.
6. CHILDS, J. F. L.
   1968. Color test for exocortis. P. 83-86 *In* Indexing procedures for 15 virus diseases of citrus trees. Agriculture Handbook No. 333 U.S. Dept. Agric., Wash., D.C.
7. COLPAN, M., J. SCHUMACHER, W. BRÜGGERMAN, H. L. SÄNGER, and D. RIESNER
   1983. Large-scale purification of viroid RNA using Cs_2 SO_4 gradient centrifugation and high-performance liquid chromatography. Anal. Biochem. 131: 257-265.
8. FUDL-ALLAH, A. E.-S. A., J. J. SIMS, and E. C. CALAVAN

1974. Indexing of exocortis virus-infected citron by using thin-layer chromatography. Plant Dis. Rep. 58: 82-85.

9. LA ROSA, ROSA, G. BOCCARDO, and M. DAVINO
   1984. L'analisi elettroforetica applicata alla diagnosi del viroide del nanismo del crisantemo. Giornate Fitopatologiche 1984, 3: (in press).
10. PALUKAITIS, P., and R. H. SYMONS
    1980. Purification and characterization of the circular and linear forms of chrysanthemum stunt viroid. J. Gen. Virol. 46: 477-489.
11. RANDLES, J. W.
    1975. Association of two ribonucleic acid species with cadang-cadang disease of coconut palm. Phytopathology 65: 163-167.
12. ROISTACHER, C. N.
    1976. Detection of citrus viruses by graft transmission: A Review, p. 175-184. *In* Proc. 7th Conf. IOCV. IOCV, Riverside.
13. ROISTACHER, C. N., E. C. CALAVAN, R. L. BLUE, L. NAVARRO, and R. GONZALES
    1977. A new more sensitive citron indicator for detection of mild isolates of citrus exocortis viroid (CEV). Plant Dis. Rep. 61: 135-139.
14. SCHUMACHER, J., J. W. RANDLES, and D. REISNER
    1983. Viroid and virusoid detection: an electrophoretic technique with the sensitivity of molecular hybridization. Anal. Biochem., (in press).