

Use of Polyacrylamide Gel Electrophoresis for Detection of Chrysanthemum Stunt Viroid in Infected Tissues

R. K. HORST, Associate Professor, and S. O. KAWAMOTO, Research Technician, Department of Plant Pathology, Cornell University, Ithaca, NY 14853

ABSTRACT

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Chrysanthemum stunt viroid (CSV) was detected in as little as 50 mg of infected *Chrysanthemum morifolium* tissue by extraction of viroid nucleic acid followed by 5% polyacrylamide gel electrophoresis (PAGE) isolation. The procedure described by Morris and Wright was modified because colored compounds in nucleic acid extracts from chrysanthemum leaves interfere with distinctness of nucleic acid bands after PAGE. CSV-infected leaf tissue was extracted with a borate-sodium sulfite, sodium lauryl sulfate, lithium chloride buffer (pH 9.0) and then treated with phenol and chloroform. Preparations were dialyzed against distilled water to remove colored compounds. Nucleic acids were stained after electrophoresis with ethidium bromide or scanned with a spectrophotometer at 260 nm. The viroid-specific RNA band could then be readily observed in gels containing nucleic acids from CSV-infected tissues.

Chrysanthemum stunt viroid (CSV), first described by Dimock (3) in 1947 and identified as a nonencapsidated low molecular weight RNA by Diener and Lawson (2) in 1973, causes severe losses in flower production in infected plants (6). Because chrysanthemum (*Chrysanthemum morifolium*) is vegetatively propagated, CSV is readily transmitted, and the best means of controlling CSV is to establish CSV-free plants through an indexing program (7). Current indexing programs generally rely on the use of bioassays that are laborious and require incubation periods of several months and extensive greenhouse space.

Procedures for extracting viroid RNA from small quantities of viroid-infected tissues followed by electrophoretic analysis of the extracts on 5% polyacrylamide gels (PAGE) have been described for detection of potato spindle tuber viroid and CSV (5,9-12). Although PAGE assay for CSV has been shown to be as reliable as bioassay, the amount of tissue required for viroid RNA extraction and the occurrence of heterogeneous compounds that obscure viroid RNA bands in polyacrylamide gels have been limiting factors. This report describes a modified procedure for PAGE assay for CSV in infected chrysanthemum tissues that is rapid, efficient, and reliable.

MATERIALS AND METHODS

Chrysanthemum 'Bonnie Jean' was inoculated with CSV by tissue implanta-

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were added along with 1 drop of 2 M sodium acetate. The preparation was mixed and incubated at -20°C for at least 30 min.

Step 7. The ethanol-dialysate mixture was centrifuged at 8,000 rpm for 20 min, and the precipitate was saved. Because the precipitate may be difficult to locate, tubes were marked before centrifugation so that the expected position of the precipitate could be readily found. The precipitate was thoroughly dried by inverting the tubes in front of a fan for 15 min.

Step 8. Precipitate was dissolved in 0.1 ml of water. Care was taken to prevent the preparation from being dispersed into small droplets around the centrifuge tube.

Electrophoresis. Five percent polyacrylamide gels were prepared according to the method of Loening (8) by combining 41.4 ml of water, 3.3 ml of 20X TAE buffer (0.02 M disodium ethylenediamine tetraacetate, 0.8 M tris [hydroxymethylaminomethane] [Trizma base], and 0.4 M sodium acetate, pH 7.6), and 22 ml of acrylamide stock (15 g of acrylamide monomer and 0.76 g of N, N'-methylenebisacrylamide [Eastman Organic Chemicals No. 5521 and No. 8383, respectively, Eastman Kodak Co., Rochester, NY], plus water to 100 ml, filtered through Whatman No. 1 qualitative filter paper). After evacuation of oxygen under vacuum, 0.68 ml of 10% ammonium persulfate and 52.5 μl of TEMED (N,N,N', N'-tetramethylethylenediamine, Bio-Rad Laboratories, Richmond, CA) were added and the gel solution was poured between glass slab gel plates (11.5 \times 14 \times 0.4 cm) with a 25-tooth comb or into Plexiglas tubes 9 cm long with an inside diameter of 6 mm. Gels were used 1 hr after pouring.

Electrophoresis was done in a slab gel cell (Aquabogue Machine and Repair Shop, Aquabogue, NY 11931) or in a Bio-Rad Model 150 A gel electrophoresis cell with 1X TAE buffer in both reservoirs. The power source (Buchler Power Supply) was set for constant-current operation at 100 V for slab gels or 6 mA per tube for tube gels. One drop of 75% RNase-free sucrose solution containing 0.5 mg per milliliter of bromophenol blue and 0.01 mg per milliliter of xylene cyanole was mixed with 0.1 ml of extract and layered on the gel surface. The dye front reached the base of the gel in about

tion (4) from CSV-infected 'Bonnie Jean'. Plants were grown in the greenhouse at 27 $^{\circ}\text{C}$ with supplemental light (16-hr photoperiod) from reflectorized lamps (Sylvania FR 96T12 CW VHO, 235 angle).

Extraction. Boiled distilled water was used when water was specified for making solutions. Extractions were carried out at 4 $^{\circ}\text{C}$ or at room temperature.

Step 1. Leaf blade tissue (1 g) was excised with a razor blade, frozen with liquid N_2 , and ground in a mortar and pestle.

Step 2. Before thawing, tissue was homogenized with 7 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 1 ml of BSSL extraction buffer (0.2 M H_3BO_3 , 12% Na_2SO_3 , 1% sodium lauryl sulfate [SDS], 2 M LiCl); the buffer was adjusted to pH 9.0 with 4 M NaOH before addition of SDS and LiCl. The BSSL buffer was heated to dissolve the SDS, then used at room temperature (21 $^{\circ}\text{C}$) while on a magnetic stirrer. The final pH was 8.0.

Step 3. Chloroform (1 ml) was added to the preparation, and the mixture was emulsified in a 16-ml polypropylene centrifuge tube with a Vortex mixer.

Step 4. The homogenate was centrifuged for 20 min at 8,000 rpm (7,900 g) in a Sorvall SS-3 centrifuge using a SM-24 rotor.

Step 5. The upper aqueous phase was immediately drawn off with a pasteur pipette and transferred to 1-cm wide dialyzer tubing, with care not to transfer phenol or interphase material. Preparations were dialyzed against 2 L of distilled water for 18 hr; the water was changed at least once during this period.

Step 6. Dialysate volume was measured with a pasteur pipette and added to a 16-ml polypropylene centrifuge tube. Two volumes of chilled (-20°C) 95% ethanol

2.5 hr; electrophoresis then was terminated.

Nucleic acids were stained with ethidium bromide (1). Gels were soaked in the stain (2 mg of ethidium bromide per 100 ml of 10^{-3} M EDTA) for 0.5 hr, which was a modification of the procedure described by Dickson (1). Stained gels were examined immediately under UV light (Mineralite lamp, Model UVS-54); if ethidium bromide appeared to be absorbed throughout the gel, which would interfere with fluorescence of CSV-RNA bands, the gel was destained for 0.5–1 hr in distilled water. Ethidium bromide-stained nucleic acids fluoresced under shortwave UV light after electrophoresis in a 3.8-mm thick slab gel. CSV could also be detected in unstained tube gels by scanning the gel spectrophotometrically at 260 nm with a Gilford Model 240 spectrophotometer equipped with a linear transport system and coupled to a Gilford 6051 recorder.

RESULTS AND DISCUSSION

The viroid-specific RNA was readily observed in gels containing nucleic acids from CSV-infected tissues stained with ethidium bromide but not in similar gels prepared from uninfected tissues. Bioassays of gel fractions containing the viroid RNA band by elution from the gels and inoculation to healthy 'Bonnie Jean' confirmed its association with CSV. Bioassays of gel fractions from PAGE assays of extracts from uninfected tissues revealed no CSV activity. Viroid-specific RNA peaks were found in scanning profiles of extracts from CSV-infected tissues but not in profiles of extracts from uninfected tissues (Fig. 1).

A more than threefold increase in recovery of CSV-RNA from CSV-infected chrysanthemum was obtained with our procedure (Fig. 1B) compared with that described by Morris and Smith (9) (Fig. 1E). In addition, CSV-RNA recovery was more efficient with BSSL extraction buffer containing LiCl cooled to 21 C (Fig. 1B–D). Amounts of CSV-RNA extracted were estimated by calculating the area under the viroid peaks in the scanning profiles, using the formula $A = h \times w$, where A = area, h = height of peak, and w = width of peak at 0.5 h. With our procedure using 21-C BSSL extraction buffer, warm BSSL extraction buffer, and warm BSSL extraction buffer minus LiCl, areas of CSV-RNA in profile scans were 52, 27, and 20 mm^2 , respectively. With the procedure described by Morris and Smith (9), however, the area of the CSV-RNA peak was 16 mm^2 . With our procedure, CSV could be detected in leaf tissue amounts as small as 50 mg from CSV-

infected chrysanthemum 'Bonnie Jean' (Table 1). CSV-RNA bands could be detected from a pooled sample of 10 chrysanthemum leaf specimens, as determined by diluting 0.5 g of CSV-infected chrysanthemum 'Bonnie Jean' with 4.5 g of healthy 'Bonnie Jean' and assaying the pooled specimen with PAGE.

Our PAGE assay for detection of viroids in small amounts of plant tissues differs from those reported by Morris and Smith (9) and Mosch et al (11) in the following ways:

1. Instead of glycine, 0.2 M H_3BO_3 is used in the extraction buffer, resulting in less discoloration in the extract from chrysanthemum tissues.
2. Instead of 3.5% NaCl, LiCl and exceptionally high concentrations (12%) of Na_2SO_3 are used in the extraction buffer; this helps provide cleaner preparations from chrysanthemum tissues.
3. Dialysis is used to remove undesirable colored compounds.
4. Phenol and extraction buffer are

Table 1. Detection of chrysanthemum stunt viroid (CSV) in CSV-infected chrysanthemum 'Bonnie Jean'

Tissue weight	CSV in polyacrylamide gels		
	PAGE assay	Dilution	PAGE assay
1.0 ^a	12/12 ^b	1.0:0 ^c	4/4 ^b
0.5	8/8	0.5:0.5	4/4
0.25	8/8	0.25:0.75	4/4
0.10	10/12	0.10:0.90	2/4
0.05	4/8	0.05:0.95	0/4
0.01	0/8	0.01:0.99	0/4

^aGrams of CSV-infected chrysanthemum 'Bonnie Jean.'

^bGels with CSV bands over number of tests.

^cRatio of grams of CSV-infected chrysanthemum 'Bonnie Jean' to grams of healthy chrysanthemum 'Bonnie Jean.'

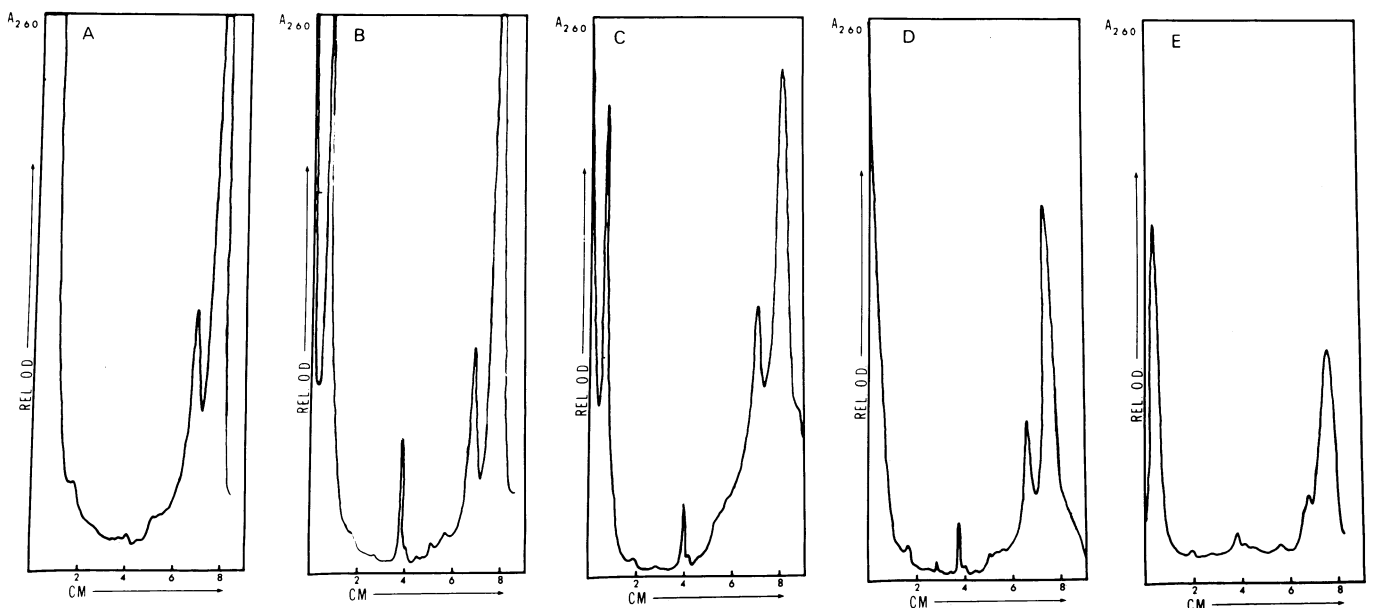


Fig. 1. Comparison of polyacrylamide gel electrophoresis (PAGE) procedures for detecting chrysanthemum stunt viroid (CSV) in CSV-infected chrysanthemum 'Bonnie Jean.' Scanning profiles at A_{260} of (A) healthy 'Bonnie Jean' using BSSL extraction buffer (0.2 M H_3BO_3 , 12% Na_2SO_3 , 1% sodium lauryl sulfate, 2 M LiCl adjusted to pH 9.0 with 4 M NaOH) cooled to 21 C, (B) BSSL extraction buffer at 21 C, (C) BSSL extraction buffer at 50 C, (D) BSSL extraction buffer minus LiCl at 50 C, and (E) Morris and Smith procedure (9).

added before tissue is thawed to prevent formation of colored compounds that interfere with visualization of RNA associated with CSV.

5. Leaf tissue is frozen with liquid nitrogen before being pulverized. This step is not necessary to recover detectable amounts of CSV but results in more uniform release of viroid RNA.

Although our procedure is insufficient for complete purification of CSV-RNA, it works very well for detection of CSV in infected chrysanthemum tissues.

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