Detection of Chrysanthemum Stunt Viroid in South Africa by Polyacrylamide Gel Electrophoresis and Bioassay

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ABSTRACT

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Chrysanthemum stunt viroid (CSV) was isolated from several commercial cultivars of *Chrysanthemum morifolium* in South Africa for the first time. Both polyacrylamide gel electrophoresis (PAGE) and bioassay were found suitable for routine detection of CSV in South African chrysanthemums. Viroid-specific RNA was isolated from infected tissues by nucleic acid extraction followed by PAGE and detected in the gels scanned at 260 nm or stained with 0.005% toluidine blue O or 0.8% ethidium bromide. A relative mobility of 0.63 of the viroid-specific RNA compared with the 5S host RNA could be used to locate viroid bands. Symptoms typical of CSV were produced by chip-budded plants of the cultivars Mistletoe, Fanfare, and Bonnie Jean maintained at 20,000 lux. Under greenhouse conditions, sap-inoculated *Senecio cruentus* consistently produced systemic symptoms typical of CSV infection.

Chrysanthemum stunt viroid (CSV) causes one of the most important diseases of ornamentals known today (14). It spreads rapidly during vegetative propagation of florist's chrysanthemums (*Chrysanthemum morifolium* Ramat), and if unchecked, the disease can reach epidemic proportions, with resultant substantial economic losses.

Distribution of cuttings by international sales has spread chrysanthemum stunt from the United States, where it was first reported (5), to Canada (25), Europe (19,24), England (6), and probably to most other chrysanthemum-growing countries.

Symptoms similar to those reported for chrysanthemum stunt (4,9,12) have become prevalent in several popular cultivars of chrysanthemum in South Africa during the past decade. By techniques of bioassay (1,3,4,7,11–13,18,20,24) and electrophoretic assay on 5% polyacrylamide gels (PAGE) (8,15,17), the causal agent has been identified as CSV.

The degree to which the spread of the disease in South Africa is controlled will depend on the efficiency of the indexing programs implemented. Bioassay and PAGE methods suitable for indexing for CSV in South Africa are described.

MATERIALS AND METHODS

Biological tests. Healthy cuttings of the *C. morifolium* cultivars Mistletoe, Fanfare, Bonnie Jean, and Good News were rooted in vermiculite under mist, planted in steam-pasteurized soil, and maintained in the greenhouse at 20–22 C under natural daylight with the dark period interrupted from 2200 to 0200 hours. When 10–20 cm tall, plants were inoculated with tissue from *C. morifolium* plants in which no virus could be detected but which showed symptoms typical of CSV. Sap inoculation (24), top grafting (10,24), approach-bottle grafting, and chip-budding (24) techniques were compared using the cultivar Mistletoe. The chip-budding technique (24), in which two wedges of candidate stem tissue were inserted in wedge shaped cuts on the stem of an indicator plant directly above and below an axillary bud and bound in place with self-adhesive bandage, was compared on Mistletoe, Fanfare, Bonnie Jean, and Good News. Immediately after inoculation, plants were transferred to a growth cabinet where, with uninoculated control plants of each cultivar, they were maintained at 21 or 28 C, 15,000 or 20,000 lux, 16-hr light period, and 85% RH for several months. Inoculated and uninoculated Bonnie Jean plants were also maintained in the greenhouse at 20–22 C and 7,000 lux.

Florist's cineraria, *Senecio cruentus* (Masson) DC., Palette, and *Verbesina encelioides* (Cav.) Benth. & Hook were grown from seed in steam-pasteurized soil in the greenhouse. Chrysanthemum leaves suspected of being CSV-infected were ground in 0.01 M sodium-potassium phosphate buffer, pH 7.0, at a ratio of 1:2 of tissue mass to buffer volume and used to inoculate the Palette and *Verbesina* seedlings. Leaves of Palette were also inoculated with chrysanthemum leaves suspected of being CSV-infected ground in an aqueous solution of 0.5% K_2HPO_4 containing 0.5% Na_2SO_3 at a ratio of 1:2 of tissue mass to buffer volume and decolorized for starch lesions according to the method described by Lawson (13). Inoculated and uninoculated plants were maintained either in the greenhouse at 20–22 C or in growth cabinets at 28 C, 20,000 lux, 16 hr photoperiod, and 85% RH.

Electrophoretic tests. Nucleic acid extractions were made from young, succulent leaves of chip-budded Mistletoe, Fanfare, and Bonnie Jean and naturally infected Marble, Polaris, Applejack, and Snowdon and used for PAGE assays. Leaves of healthy Mistletoe, Fanfare, Bonnie Jean, and Marble were used as controls.

Distilled water boiled for 10 min and analytical-grade chemicals were used to prepare all solutions. Glassware was sterilized by boiling for 10 min in distilled water, and surgical gloves were worn at all times. Extraction of nucleic acids and PAGE were carried out at room temperature unless otherwise specified. The following procedure for the extraction of nucleic acids was developed by modification of the method of Mosch et al (17):

Step 1. Four grams of succulent leaf tissue was cut into fine strips and homogenized with an Ultra Turrax (Janke and Kunkel, IKA-WERK) for 60 sec with 4 ml extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, 1.0% sodium lauryl sulphate adjusted to pH 9.5 with 5 M NaOH) at 23 C, 8 ml chilled water-saturated phenol containing 0.1% 8-hydroxyquinoline, 8 ml chilled chloroform:pentanol (25:1, v/v), and two drops of chilled 2-mercaptoethanol.

Step 2. The homogenate was transferred to 30-ml Corex tubes and centrifuged for 15 min at 8,000 *g* and 4 C.

Step 3. The clear, golden aqueous phase was drawn off, the volume was measured, and it was transferred to a 30-ml Corex tube. One-quarter volume chilled 10 M LiCl was added and the tube was shaken vigorously for a few seconds, then incubated on ice for 2 hr.

Step. 4. The white precipitate was pelleted by centrifuging for 15 min at 8,000 *g* and 4 C.

Step 5. The supernatant was drawn off, transferred to 240-mm-wide dialysis tubing, and dialyzed with gentle stirring against 2 L chilled, distilled water at 4 C. The water was replaced after 2 hr, after which dialysis continued for an additional

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16 hr.

Step 6. The volume of the olive to gold dialyzate was measured and transferred to 30-ml Corex tubes. Two volumes of chilled absolute ethanol and two drops of 2 M sodium acetate were added and the tubes were incubated at −20 C for 6 or 24 hr.

Step 7. The nucleic acid precipitate was collected by centrifuging for 15 min at 8,000 *g* (4 C) and was resuspended in 0.45 ml 1× PAGE buffer [0.04 M tris (hydroxymethylaminomethane), 0.02 M sodium acetate, and 0.001 M disodium ethylenediamine tetraacetate (EDTA), pH 7.2] by gentle stirring on ice.

Step 8. The partially purified extract was centrifuged for 8 min at 8,000 *g* and 4 C. The supernatant was collected and either stored at −20 C or loaded immediately onto the gels.

The extraction procedure required 2 days and PAGE was carried out on the third day. Five percent polyacrylamide gels were prepared by combining 7.5 ml acrylamide solution (1.5 g acrylamide and 0.0375 g N,N′-methylenediacrylamide in 10 ml 1× PAGE buffer filtered through Whatman No. 1 qualitative filter paper), 4.5 ml 5× PAGE buffer, 3.75 ml 1% ammonium persulfate, and 6.75 ml distilled water. Oxygen was removed from the solution for 5 min under vacuum, 11 µl TEMED (N,N,N′,N′-tetramethylethylenediamine) was added, and the gel solution dispensed into 12 glass tubes (90 × 6 mm). The gels were polymerized at room temperature overnight.

Electrophoresis was performed at room temperature in a Biorad model 150A gel electrophoresis cell with 1× PAGE buffer. The gels were prerun at 3 mA per tube for 15–30 min. Four drops of 75% saccharose solution containing 0.5 mg/ml bromophenol blue (8) was mixed with each 0.45-ml nucleic acid extract, and samples of 50, 100, and 150 µl were applied to the gel surface with an autopipette. The gels were electrophoresed at 3 mA per tube for 15 min and then 6 mA per tube until the bromophenol blue marker band was 5 mm from the bottom of the gel (2.25–2.5 hr).

Each gel was scanned at 260 nm with a Beckman model 35 spectrophotometer and stained overnight in 0.1% (16) or 0.005% toluidine blue O in water with a change of stain after 30 min or rinsed in distilled water and stained in ethidium bromide (8 mg/1,000 ml 0.001 M EDTA) for 15 min (23). Gels stained in toluidine blue were destained in distilled water and examined over a white background. The positions of the nucleic acid bands were measured and compared with the nomogram published by Mosch et al (17). Gels stained in ethidium bromide were examined under shortwave ultraviolet (UV) light (CAMAG universal UV lamp, 254 nm) and compared with the nomogram (17).

RESULTS

Comparison of inoculation techniques. Transmission to Mistletoe was achieved with sap inoculation and all three graft techniques. At 21 C and 20,000 lux, approach-grafted plants expressed symptoms within 5–8 wk, top-grafted plants within 4 wk, and sap-inoculated plants within 5 wk; symptoms consisted of small (1–2 mm) yellow spots and were not expressed by all the plants. In contrast, all the chip-budded plants grew vigorously and produced clearly defined, irregular chlorotic spots (1–4 mm), with some leaf distortion and curling within 3–4 wk.

Comparison of chrysanthemum indicators. Fanfare, Mistletoe, and Bonnie Jean plants showed distinct leaf symptoms within 10 wk but Good News plants showed only dwarfing, even after 5 mo. In all cases, symptoms were first expressed on the shoot growing from the bud between the implanted tissue chips. Light intensity affected the length of the latent period before symptom expression. When incubated at 28 C, Bonnie Jean plants produced symptoms more rapidly at 20,000 lux than at 15,000 lux, whereas Mistletoe and Fanfare plants expressed symptoms only slightly faster at 20,000 lux than at 15,000 lux (Table 1). Bonnie Jean plants maintained in the greenhouse were symptomless.

Fanfare showed distinct light green veinclearing followed by downcurling of the leaves from the midrib and production of pale, upright, young leaves. Mistletoe developed irregular yellow spots (1–4 mm) and some leaf distortion. Bonnie Jean did not express symptoms consistently. Some plants developed faint veinclearing and small yellow spots (1–2 mm); others developed large, irregular yellow spots (1–6 mm), and a few did not develop symptoms.

Cineraria and *V. encelioides* as indicators. No starch or chlorotic local lesions were observed on Palette leaves inoculated and decolorized as described by Lawson (13); however, these plants developed systemic shortening of the internodes, dwarfing, curling of leaves, and flower malformation after 2 mo in the greenhouse. Cineraria plants inoculated with inoculum containing 0.01 M phosphate buffer, pH 7.0, and maintained in the greenhouse developed local chlorotic spots (1–2 mm) after 26 days and systemic symptoms after 10 wk. In the growth cabinet, plants grew weakly and did not develop symptoms. No symptoms were obtained on inoculated *V. encelioides* plants maintained in either the greenhouse or growth cabinets for 3 mo.

Electrophoretic tests. Gels containing nucleic acid extracts from naturally and experimentally infected tissues of all the cultivars tested possessed an RNA species not present in gels containing similar extracts from healthy tissues. This extra RNA occurred as a distinct peak of absorbance at 260 nm (Fig. 1) or stained band (1 mm wide) in the central region of gels containing 50, 100, or 150 µl of nucleic acid extract. The relative mobilities of nucleic acid species in comparison to the mobility of the 5S host RNA were calculated as follows:

$$R_m = \frac{\text{distance from top of gel to NA peak/band}}{\text{distance from top of gel to 5S peak/band}}.$$

The R_m of the extra RNA species was 0.63 ± 0.01 when calculated from either scanning profiles or toluidine blue-stained gels. The extra RNA band in gels stained with either toluidine blue or ethidium bromide intersected the R_m 0.61 line of the nomogram (17).

The 7S RNA (21) formed a clearly visible band (1 mm wide) slightly below the extra RNA species in all the gels stained with ethidium bromide and most of those stained with toluidine blue, but it yielded only a very small peak in scanning profiles (Fig. 1D). The R_m of the 7S RNA calculated from scanning profiles was 0.72 ± 0.02, and from 0.005% toluidine blue-stained gels, it was 0.70 ± 0.02.

In toluidine blue- and ethidium bromide-stained gels, one or two additional narrow bands were visible occasionally between the 7S and 5S bands. The R_ms of these bands were 0.85 ± 0.02 and 0.93 ± 0.01.

Gels containing 50 µl of nucleic acid extract yielded scanning profiles with minimal background absorbance from colored compounds and clearly defined peaks (Fig. 1A,B), and they stained so that the nucleic acids appeared as densely stained bands on a faintly stained background. The nucleic acid species were most easily located on these gels. Gels containing 100- and 150-µl samples yielded scanning profiles with plateaus of absorption from colored compounds between the DNA and 5S RNA peaks (Fig. 1C,D). These plateaus partly obscured the peak of the extra RNA

Table 1. Days until symptom development in *Chrysanthemum morifolium* cultivars after chip-bud inoculation with chrysanthemum stunt viroid-infected tissue

Temperature (C)	Light intensity (lux)	Cultivar		
		Mistletoe	Fanfare	Bonnie Jean
21	7,000	...[a]	...[a]	No symptoms
28	15,000	28–50	16–29	70
	20,000	27	15–21	20–55

[a] Not tested.

species in scans of gels containing samples from infected tissues. Fairly dense background staining in the central area of stained gels corresponded with the plateaus of absorbance in the scanning profiles and made location of the nucleic acid band more difficult than with the 50-μl sample gels.

At a concentration of 0.1%, toluidine blue O stained the gels very densely and the nucleic acid bands could only be detected after destaining for several days. Efficient staining was obtained with 0.005% toluidine blue. The bands could be seen clearly after 1–2 hr of destaining.

DISCUSSION

CSV has been determined present in South African chrysanthemums. This determination was based on 1) symptoms exhibited by plants in commercial plantings, 2) bioassays on chip-budded Mistletoe, Fanfare, and Bonnie Jean indicator plants and sap-inoculated *S. cruentus* plants, and 3) the presence of an RNA species with a R_m 0.63 in 5% polyacrylamide gels of nucleic acids from infected tissues. This is the first report of CSV in South Africa.

The most rapid and efficient method of bioassay for CSV was by chip budding to young Mistletoe and Fanfare plants. High light intensity was conducive for symptom production (1,7), and Mistletoe, Fanfare, and Bonnie Jean expressed symptoms faster at 20,000 lux than at 15,000 lux (Table 1).

Local lesions on cineraria (2,3,7,12,13) and *V. encelioides* (7) do not reliably detect CSV. This was confirmed for South African conditions; however, cineraria consistently produced systemic symptoms of CSV infection under greenhouse conditions (2). These symptoms were a reliable indication of the presence of CSV, and in cases where controlled-environment cabinets were unavailable, could be used to detect the viroid.

The PAGE assay method was found suitable for detection of CSV in all cultivars tested. The assay could be completed in 3–4 days, depending on the method used to detect the viroid-specific RNA.

Use of boiled water and glassware and an extraction buffer cooled to a temperature just above that at which the SDS solidified as recommended by Horst and Kawamoto (8) greatly improved the separation of the nucleic acids and the detectability of the viroid-specific RNA in the gels. Removal of colored compounds by dialysis was also found essential for efficient detection of the viroid-specific RNA.

The duration of the concentration in ethanol used by other workers varied from 30 min (22) to overnight (15). In this study, the 6- and 24-hr periods gave equally good results.

In contrast to the findings of Mosch et al (17), variation in the degree of contamination with colored compounds of extracts from different cultivars did not occur; however, the larger the sample of nucleic acid extract applied to the gel, the greater was the degree of absorbance and staining due to colored compounds in the central area of the gel. Application of a small (50-μl) sample to the gel minimized interference from colored compounds with scanning or staining. Staining was found to be a more sensitive method for detecting viroid-specific RNA than scanning at 260 nm. Both toluidine blue and ethidium bromide stained the host and viroid-specific nucleic acid bands well. Staining with ethidium bromide was quicker, and the bands were usually clearer than with toluidine blue; however, toluidine blue-stained gels were easier to view, measure, and compare with the R_m nomogram than ethidium bromide-stained gels, which had to be viewed under UV light. Calculation of the R_m of a nucleic acid species against the 5S host RNA facilitated its location. This was particularly important when determining whether a single peak or band in the central region of the gel was viroid-specific or 7S host RNA. The nomogram (17) was very useful for rapid identification of viroid-specific RNA bands in stained gels.

Both bioassay and electrophoretic assay (PAGE) are suitable methods for indexing for CSV. The method chosen for use in any particular situation will depend on the facilities available and the speed with which the result is required.

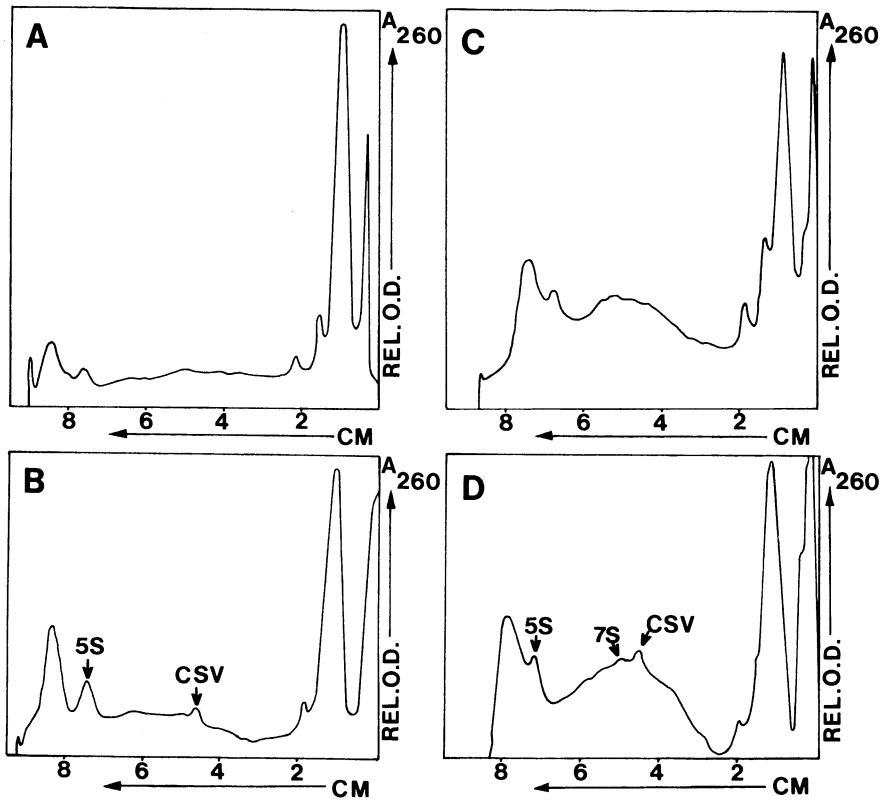

Fig. 1. Detection of chrysanthemum stunt viroid (CSV) in unstained 5% polyacrylamide gels. Scanning profiles at A_{260} of gels containing 50 μl of nucleic acid extract **(A)** from healthy tissue and **(B)** from CSV-infected chrysanthemum tissue and 150 μl of nucleic acid extract **(C)** from healthy tissue and **(D)** from CSV-infected chrysanthemum tissue.

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