

Isolation and Analysis of Double-Stranded RNA from Virus-Infected Plant and Fungal Tissue

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ABSTRACT

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A simple, rapid method for the isolation of double-stranded RNA (dsRNA) from virus-infected plant and fungal tissues provides a new approach to virus detection and identification. Diseased tissue was phenol-extracted to isolate cellular nucleic acids, and viral dsRNA was selectively purified from other nucleic acids by binding to cellulose powder in 15% ethanol either in small columns or by a batch procedure. The product was analyzed first by gel electrophoresis and then by ribonuclease treatment to

identify dsRNA. The method permits rapid and efficient isolation and analysis of dsRNA from small amounts (1–10 g) of tissue and from multiple samples using small amounts (0.1–2.5 g) of cellulose powder. Successful isolation of dsRNA does not depend on the type of tissue processed and the method therefore is potentially useful for the study of RNA virus replication and for detection and diagnosis of virus infections directly from the infected host tissues.

Additional key word: disease detection.

The isolation and properties of viral-specific double-stranded RNA (dsRNA) from tissue infected with RNA viruses have been well documented (12). Analysis has most often involved lengthy isolation procedures that yield small quantities of dsRNA. The quality of dsRNA has been uncertain for some viruses (2,13). The need for better recovery and quality of dsRNA has resulted in the development here of a simple and rapid column or batch procedure for the isolation of dsRNA. It has been used to isolate dsRNA from several systems infected with either single-stranded RNA (ssRNA) or dsRNA viruses. Preliminary reports have been published (3,10).

The method is based on the affinity of cellulose powder for nucleic acids (5) and, specifically, the adsorption of dsRNA at ethanol concentrations of 15%. The product is analyzed by gel electrophoresis and dsRNA then is identified by ribonuclease treatment. The method permits rapid, efficient isolation and analysis of dsRNA from relatively small amounts of tissue from multiple samples, and success appears to be independent of the type of tissue processed. Consequently it is a potentially useful tool for the study of RNA virus replication and for detection and diagnosis of virus infections directly from host tissue.

MATERIALS AND METHODS

Virus culture. Bromegrass mosaic virus, the cowpea strain of southern bean mosaic virus and the cherry strain of tomato bushy stunt virus were obtained from R. I. Hamilton of Agriculture Canada, Vancouver, and maintained in *Hordeum vulgare*, *Vigna sinensis* and *Nicotiana clevelandii*, respectively. Tobacco necrosis virus with satellite virus, turnip yellow mosaic virus (TYMV), and the cowpea strain of tobacco mosaic virus were obtained from frozen isolates at Berkeley and propagated in *Nicotiana tabacum* L. Xanthi, *Brassica chinensis* cv. Michili and *V. sinensis*, respectively. Isolations of alfalfa mosaic virus and cucumber mosaic virus were made in California and the viruses were maintained in *N. tabacum*.

Inoculated plants were maintained in the greenhouse, harvested 7–14 days after inoculation, and stored frozen until use. A mycovirus containing culture of *Penicillium chrysogenum* Thom (ATCC 9480) was obtained from B. Castanho, University of California, Davis. The fungus was grown in potato dextrose broth for 2 wk, at which time the mycelium was collected by filtration and stored frozen. Two strains of *Ustilago maydis*, the P1 killer strain that carries a viruslike particle containing dsRNA and the P2 nonkiller strain that does not (9,16) were grown as shake cultures

at 25 C for 48 hr in liquid complete medium (14). The dsRNA of P1 strain has been characterized after preparation from tissue by a more elaborate method that did not involve cellulose powder (9). Both a virulent and a hypovirulent strain of *Endothia parasitica* were grown as static cultures at 28 C for 7 days as described elsewhere (3). *U. maydis* and *E. parasitica* tissue was washed in 100 ml of extraction buffer and was not frozen before extraction.

Nucleic acid extraction. Nucleic acids were extracted as described previously (11). Fresh or frozen tissue (10 g or less) was homogenized at low speed in a blender for 1 min or with a mortar and pestle in the following mixture of buffer and solutions: 10 ml of GPS buffer (0.2 M glycine, 0.1 M Na₂HPO₄, 0.6 M NaCl, pH 9.5), 1 ml of 10% SDS, 0.1 ml of mercaptoethanol, 10 ml of water saturated phenol (containing 0.1% 8-hydroxyquinoline) and 10 ml of chloroform-pentanol (25:1).

Mycelium of *U. maydis* and *E. parasitica* was homogenized with GPS buffer and 20 g of glass beads in a Braun homogenizer for 4 min. The other reagents were then added and the mixture was occasionally shaken to form and maintain an emulsion for 30 min on ice. Homogenates were centrifuged at 8,000 g for 20 min at 4 C and the aqueous phase containing cellular nucleic acids was recovered.

Purification of dsRNA. The procedure of Franklin (5), used by Jackson et al (8), German and de Zoeten (6) and Dodds et al (4) for isolating plant viral dsRNA was modified and shortened. Two procedures were compared, both of which had the following features: Replicative intermediate and replicative form were not separated in an attempt to recover as much dsRNA as possible. The interaction of sample and cellulose was in the presence of 15% rather than higher ethanol concentrations. At this ethanol concentration, dsRNA is the only major class of nucleic acid bound to cellulose (5). The amount of cellulose powder used was reduced to a level sufficient to bind only the relatively small amount of dsRNA in the sample.

Method 1. This was developed in Connecticut and used on *U. maydis* and *E. parasitica*. The cellular nucleic acids were collected from the aqueous phase by centrifugation after ethanol precipitation, resuspended in and dialyzed against STE buffer (0.1 M NaCl, 0.05 M Tris, 0.001 M Na₂ EDTA, pH 7.0), adjusted to 15% ethanol (STE/15% ethanol), and passed through a 10-ml (2.5 g dry weight) column of chromatographic cellulose powder (Whatman CF-11 cellulose) equilibrated with STE/15% ethanol. The charged column was washed with 80 ml of STE/15% ethanol and then the retained dsRNA was eluted with 15 ml of STE buffer without ethanol.

Method 2. Developed in California, this even simpler method, in addition, avoids the concentration and dialysis of nucleic acids that was unnecessary and sometimes detrimental to maximum recovery of dsRNA. The aqueous phase was adjusted to 15% ethanol by addition of absolute ethanol with stirring. Cellulose powder (Whatman CF-11 cellulose or Biorad Cellex N-1) without pretreatment, was then added at a rate of 0.25 g/20 ml of supernatant. The mixture was gently shaken, on ice, for 10 min and then centrifuged at 6,000 g for 10 min. The supernatant was discarded and the cellulose pellet was resuspended in 5 ml of STE/15% ethanol. The cellulose was then placed in a small column and washed free of ssRNA with 60 ml of STE/15% ethanol at room temperature. The dsRNA was eluted from the cellulose by the addition of 5 ml of STE buffer without ethanol. Traces of DNA were removed from the dsRNA by adding $MgCl_2$ to a concentration of 0.03 M and DNase (DNase I from Sigma) to a concentration of 10 $\mu g/ml$ and incubating the mixture for 20 min at 30 C. The dsRNA prepared by either method was concentrated by ethanol precipitation and stored at -20 C .

Gel electrophoresis. The dsRNAs were analyzed by electrophoresis in 2.4 or 5% polyacrylamide gel in 0.04 M Tris, 0.02 M sodium acetate, 0.001 M Na_2EDTA , pH 7.2, in the absence of SDS. Electrophoresis was performed in cylindrical gels ($0.6 \times 10\text{ cm}$) at 75 V, 6mA per gel. Samples for electrophoresis were dissolved in 200 μl of 1:5 concentration electrophoresis buffer with 10% sucrose and 0.001% bromophenol blue. The gels were analyzed by scanning at 280 nm with an ISCO Gel Scanner. Alternately, the nucleic acid was fixed by soaking the gels in 0.2% hexadecyltrimethyl-ammonium bromide followed by thorough washing with distilled water and staining in 0.1% toluidine blue 0 in water. Gels were destained with water and scanned at 620 nm. The concentration of the dsRNA was estimated by planimetry of the gel scans with reference to a standard curve prepared from known concentrations of TYMV dsRNA.

RESULTS

dsRNA from *U. maydis* and *E. parasitica*. The results for *U. maydis* P2 (nonkiller) and P1 (killer) using Method 1 are shown in Fig. 1. The dsRNA sample applied to each gel was the amount

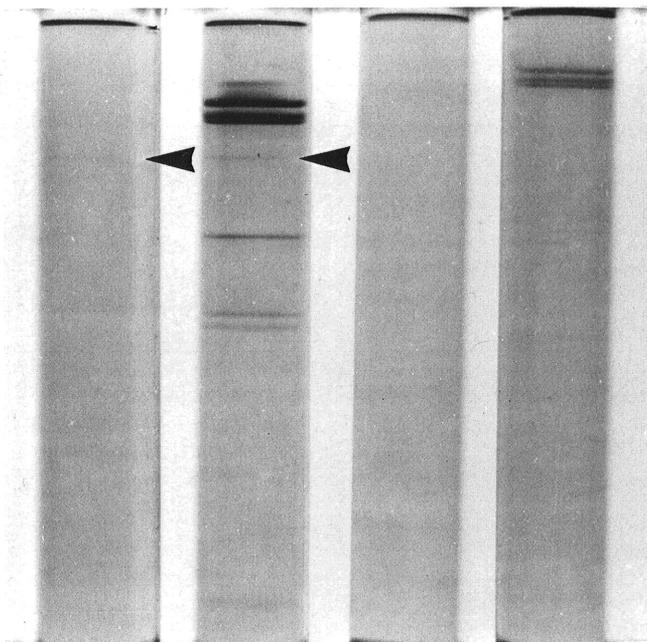


Fig. 1. Polyacrylamide gels showing double-stranded RNA prepared from fungal mycelium by method 1. The RNA was electrophoresed into 5% gels for 6 hr at 6 mA per gel and the gels were stained with 0.1% toluidine blue O. The gels contain nucleic acid extracted from (left to right): *Ustilago maydis* P2 (nonkiller); *U. maydis* P1 (killer); *Endothia parasitica*, virulent, and *E. parasitica*, hypovirulent. Note the minor component (arrowed) common to both strains of *U. maydis*.

purified from 0.4 g of tissue. The pattern of dsRNA components from P1 is similar to that reported by Koltin and Day (9) who purified dsRNA by the method described by Vodkin et al (15), which relies on the use of lithium chloride and enzyme digestion to remove all other nucleic acids. In this study the single 2.6×10^6 component of Koltin and Day (the second highest molecular weight band) was resolved as two distinct components. We detected an additional very minor component with a mobility between that of the 2.6 and 0.94×10^6 components of Koltin and Day. This component is of some interest because it also was detected in the P2 nonkiller strain used as the virus free control. No other components were detected in P2.

The results for *E. parasitica* using Method 1 also are shown in Fig. 1. The virulent strain contained no detectable dsRNA, but the hypovirulent strain contained three dsRNA components. Virulent and hypovirulent strains of *E. parasitica* were analyzed in detail and reported elsewhere (3).

Comparison of dsRNA isolation procedures. The results for *U. maydis* dsRNA prepared by Method 1, compared with another method (15), indicate that, similar results can be obtained using CF-11 to isolate dsRNA. The CF-11 cellulose method described by Jackson et al (8) for isolating dsRNA from plant tissues involves extensive purification of the cellular nucleic acids and two cycles of

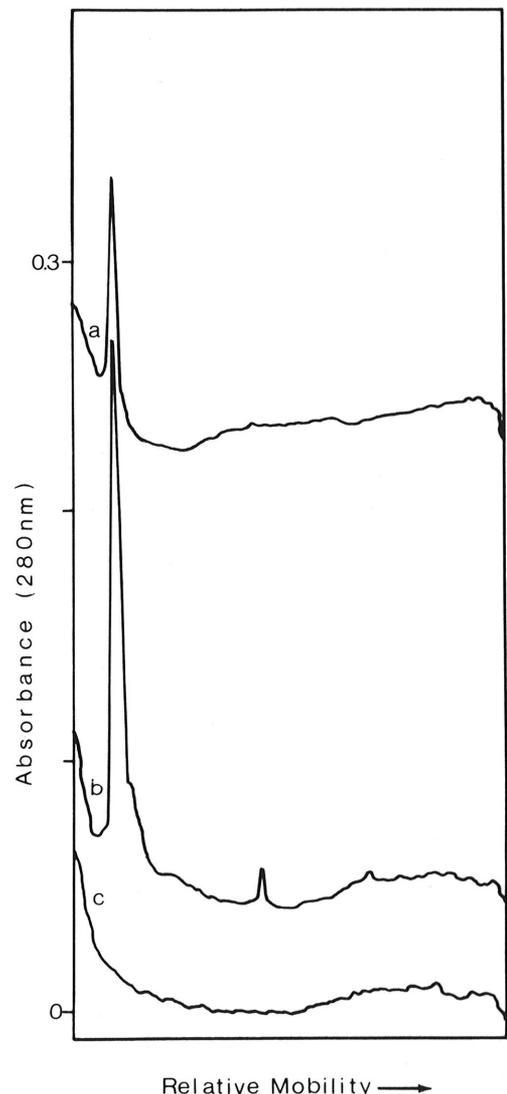


Fig. 2. Scanning profile of 2.4% polyacrylamide gels showing double-stranded RNA of turnip yellow mosaic virus prepared from 5 g of infected leaf tissue. a, Isolation by the method of Jackson et al (9); b, Isolation by method 2; c, Isolation from 10 g of healthy tissue by method 2. The RNA was electrophoresed at 75 V, 6 mA per gel for 4 hr at 15 C.

cellulose chromatography. Their procedure was compared with the shorter batch method (Method 2) using several different types of virus-infected tissue. Equivalent amounts of TYMV-infected tissue were processed for dsRNA by the two methods and electrophoresed on gels (Fig. 2). The shorter batch procedure consistently gave 40–80% greater recovery of TYMV dsRNA for all types of tissue tested, and the quality of the dsRNA was comparable as judged by gel electrophoresis. Table 1 summarizes the recovery by the two methods of dsRNA from plant and fungal tissues infected with three different viruses. It is evident that the simpler procedure, Method 2, consistently gave equal or better recoveries of dsRNA than the Jackson method.

Larger amounts of dsRNA are to be expected in fungal tissue infected with a dsRNA virus than in plant tissue infected with a ssRNA virus, and the small amounts of cellulose used in Method 2 may lead to incomplete recovery of dsRNA from infected fungal tissue. To test this possibility dsRNA was prepared from 2 g of hypovirulent *E. parasitica* by Methods 1 and 2. Method 1 yielded a greater quantity of dsRNA (25 µg) than did Method 2 (12 µg). The yield by Method 2 was increased to 20 µg when the amount of cellulose powder used was increased from 0.25 to 0.75 g. The highest yield of 40 µg was obtained by adjusting the nucleic acid extract in GPS to 15% ethanol, a feature of Method 2, and then passing the solution through a 2.5-g cellulose column equilibrated with STE/15% ethanol, a feature of Method 1.

Conditions for optimal recovery of dsRNA. The adsorption capacity of different grades of chromatographic cellulose powder for 20 µg of dsRNA in 2 ml of either GPS or STE buffer over a range of ethanol concentrations was compared to determine the optimal conditions for recovery of the dsRNA using Method 2 (Table 2). The most efficient recovery of dsRNA was obtained from the high salt, high pH GPS buffer in 15% ethanol. Higher ethanol concentrations did not improve recovery and were avoided because the binding of ssRNA is favored above 20% ethanol (5). The binding of

ssRNA was eliminated at 10% ethanol in GPS buffer, but the recovery of dsRNA was also somewhat reduced. Several brands of chromatographic cellulose were compared and all were found to bind dsRNA efficiently from solutions of 15% ethanol. Finer grades such as Biorad Cellex N-1 had greater binding capacity (20–25 µg/100 mg of cellulose) than did coarser grades such as Whatman CF-11 cellulose (5 µg/100 mg of cellulose), but the very fine grades had slow flow rates that increased the washing time during the 15% elution step. Biorad Cellex N-1 displayed the best combination of capacity and flow rate and was used in all subsequent experiments.

Temperature of incubation had little effect on the binding capacity of the cellulose for dsRNA (Table 2, h and i); therefore the colder temperature was preferred to preserve the integrity of the nucleic acids. However, temperature did have a marked effect on the efficiency of removal of ssRNA (2 M LiCl insoluble, RNase sensitive fraction) from the cellulose during the 15% elution step. Removal of ssRNA was more readily accomplished at room temperature.

DNA in the dsRNA fraction. dsRNA was detected only from virus-infected tissue. Detectable amounts of dsRNA were never isolated from healthy tissue. However, healthy and infected tobacco tissue (*N. tabacum* L. 'Xanthi' and 'Xanthi NC' and *N. clevelandii*) consistently yielded a species of nucleic acid with a mobility during electrophoresis equivalent to dsRNA of 3.0×10^6 molecular weight (Fig. 3). This species was sensitive to treatment with DNase and was concluded to be an unusual species of DNA because the great majority of the cellular DNA did not bind to the cellulose. The significance of this DNA was not further evaluated but its detection indicates that the dsRNA fraction should be treated with DNase before analysis.

Confirmation of dsRNA structure. The double-stranded nature of an RNA can be demonstrated by hyperchromicity studies, base composition analysis, density in cesium sulfate, or resistance to ribonuclease digestion. The first three methods are not easily appli-

TABLE 1. Recovery of double-stranded RNA from virus-infected tissues by the procedure of Jackson^a and by Method 2

Tissue type	µg of dsRNA isolated per gram of tissue (fresh weight)					
	Jackson method			Method 2		
	1 ^b	2	3	1	2	3
TYMV ^c -infected Chinese cabbage	0.52	0.55	0.75	0.78	0.92	1.02
AMV ^d -infected tobacco	0.11	0.15	0.09	0.15	0.26	0.17
<i>Penicillium chrysogenum</i>	1.4	0.9	1.8	2.5	1.9	2.8

^aJackson, A. O. et al 1971. *Virology* 48:182-191.

^bData is for three separate experiments; 5 g of frozen plant tissue and 1 g of *P. chrysogenum* tissue was extracted. Recovery of dsRNA was calculated from planimetry of gel scans recorded at 280 nm.

^cTYMV = turnip yellow mosaic virus.

^dAMV = alfalfa mosaic virus.

TABLE 2. Adsorption capacity of cellulose for double-stranded (ds) RNA

Buffer	Treatment ^a				% dsRNA recovered ^b			
	Ethanol concentration (%)	Cellulose amount (g)	Cellulose Grade	Temperature (C)	Turnip yellow mosaic virus dsRNA		Penicillium chrysogenum virus dsRNA	
					1 ^c	2	1	2
a) GPS	15	0.5	CF-11	4	80	78	76	78
b) GPS	15	0.1	CF-11	4	40	49	35	42
c) GPS	15	0.5	Cellex	4	85	82	71	82
d) GPS	15	0.1	Cellex	4	82	76	79	75
e) GPS	20	0.1	Cellex	4	88	91	72	75
f) GPS	10	0.1	Cellex	4	56	62	42	49
g) STE	15	0.1	Cellex	4	68	57	51	61
h) GPS	15	0.1	Cellex	25	85	79	68	72
i) GPS	15	0.1	Cellex	4	91	81	75	79

^a10 µg of dsRNA in 2 ml of buffer adjusted to the ethanol concentration was incubated with the amount and grade of cellulose, collected by centrifugation, and eluted with STE buffer.

^bRecovery calculated from the micrograms of dsRNA detected by gel electrophoresis compared with the amount of dsRNA in the initial solution.

^cTwo separate experiments performed.

cable to small quantities of RNA, and sample digestion by RNase before electrophoresis gave poor results (Morris and Dodds, *unpublished*). Figure 4 illustrates the unambiguous distinction between single and double stranded RNA when the RNase digestion is performed after electrophoresis on the RNA species in the gel. The gels were incubated in either distilled water or 0.3M NaCl after electrophoresis and scanned at 280 nm. Ribonuclease was added to the gels in 10-ml test tubes at a concentration of 50 $\mu\text{g}/\text{ml}$; the gels were incubated at 30 C and then rescanned several times after RNase addition. Single-stranded BMV-RNA was readily digested and diffused out of the gels after 2-hr incubation with RNase in either distilled water or 0.3M NaCl (Fig. 4, b and d). However, dsRNA was unaffected after 2 hr of RNase treatment in 0.3M NaCl (Fig. 4, h) and still detectable after 24 hr of incubation (Fig. 4, i). The dsRNA was eliminated by RNase treatment in distilled water (Fig. 4, f). The same experiments were performed on other single-stranded and double-stranded viral RNA's with similar results. This method allows for sensitive detection and confirmation of the presence of dsRNA in the gel profile.

Identification of plant viruses by dsRNA isolation. Gel electrophoresis can be used to determine the number and size of the dsRNA species isolated from virus-infected tissue. The gel profile of the isolated dsRNA should be characteristic of the infecting virus and therefore could be useful for identification as well as detection of the virus.

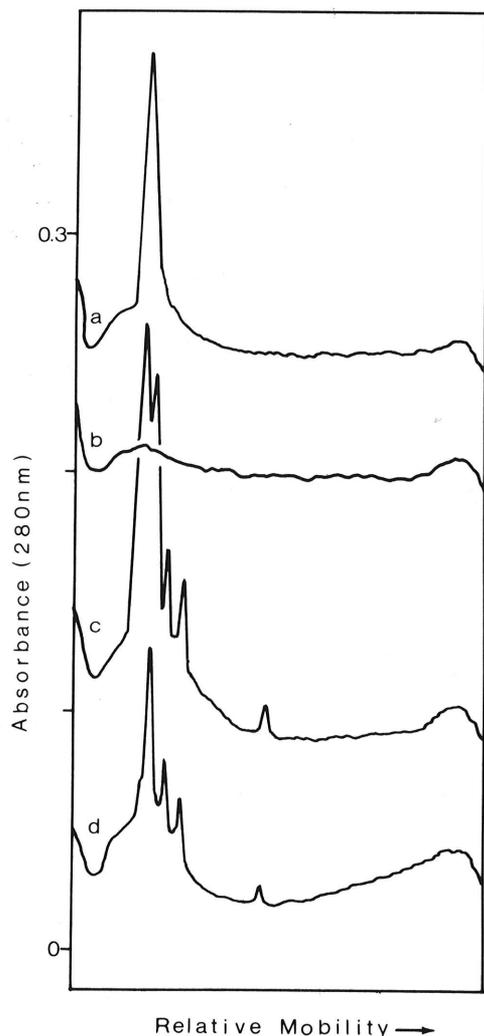


Fig. 3. Scanning profiles of 2.4% polyacrylamide gels showing nucleic acids isolated from tobacco by method 2. **a**, from healthy tobacco; **b**, from healthy tobacco, treated with DNase; **c**, from tobacco infected with alfalfa mosaic virus (AMV); and **d**, from AMV-infected tobacco, treated with DNase. Conditions for electrophoresis as in Fig. 2.

Plant tissue infected with several different viruses was processed by Method 2 and the dsRNA products were analyzed by gel electrophoresis (Fig. 5). The tissues processed were: Chinese cabbage

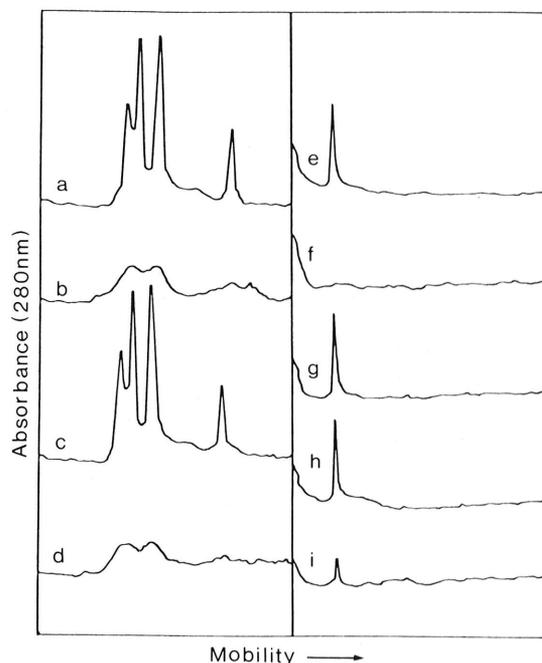


Fig. 4. Scanning profiles of 2.4% polyacrylamide gels of bromegrass mosaic virus (BMV) single-stranded (ss) RNA and turnip yellow mosaic virus (TYMV) double-stranded (ds) RNA 2 hr after incubation of the gels with and without 50 $\mu\text{g}/\text{ml}$ of ribonuclease. **a-d**, BMV-ssRNA, 2 hr after electrophoresis with the gel incubated in **a**, distilled water; **b**, distilled water and RNase; **c**, 0.3 M NaCl; and **d**, 0.3 M NaCl and RNase. **e-h**, TYMV-dsRNA, 2 hr after incubation in **e**, distilled water, **f**, distilled water and RNase, **g**, 0.3 M NaCl, **h**, 0.3 M NaCl and RNase and **i**, TYMV-dsRNA 24 hr after incubation in 0.3 M NaCl and RNase. Conditions for electrophoresis as in Fig. 2.

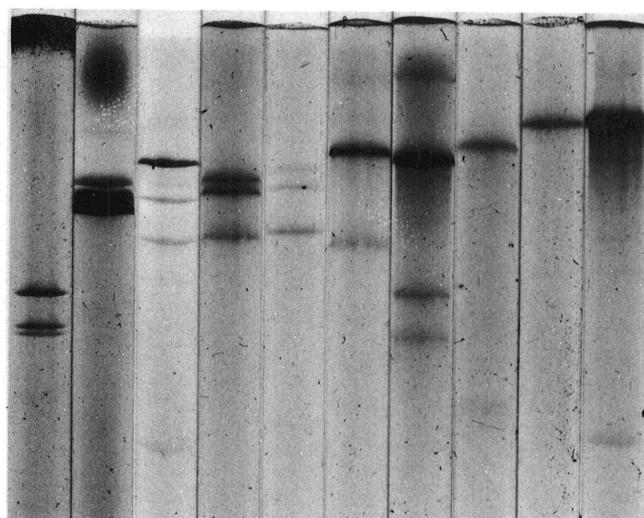


Fig. 5. Polyacrylamide gels (2.4%) showing double-stranded (ds) RNA extracted from virus-infected tissues by method 2. The gels were stained with 0.1% toluidine blue O after treatment with RNase in 0.3 M NaCl. The gels contain (from left to right) dsRNA of an unknown rose virus, *Penicillium chrysogenum*, alfalfa mosaic virus, cucumber mosaic virus, bromegrass mosaic virus, tobacco necrosis virus and satellite virus, southern bean mosaic virus, tobacco mosaic virus, and turnip yellow mosaic virus. Conditions for electrophoresis as in Fig. 2.

infected with TYMV, cowpea infected with the cowpea strains of tobacco mosaic virus and of southern bean mosaic virus, tobacco tissue infected with both tobacco necrosis virus and satellite virus, tomato bushy stunt virus, cucumber mosaic virus and alfalfa mosaic virus; barley infected with bromegrass mosaic virus; virus infected *P. chrysogenum*, and rose tissue infected with an unknown virus. The dsRNA profile were quite characteristic for each of the viruses tested (Fig. 5). These results confirm that the method is useful for the detection and identification of RNA viruses. Presence of dsRNA in rose is an example of the detection of a possible virus infection in tissue that has failed to yield infectious inocula by standard virus isolation procedures (T. J. Morris, unpublished).

DISCUSSION

A method has been described for the rapid, efficient isolation of dsRNA from plant leaves of various textures and fungal mycelium. Successful isolation of dsRNA was independent of the type of tissue used and the method should give good results with other tissue types. In addition, a sensitive *in situ* RNase digestion test has been developed for unambiguous evaluation of the double-stranded nature of the isolated nucleic acids. The method may prove useful for detection and identification of RNA virus infections.

The batch method (Method 2) works well for all kinds of tissues including those that produce viscous extracts that would not pass readily through a column. The amount of cellulose used, 0.25 g, is sufficient to bind a quantity of dsRNA that can be detected by gel electrophoresis, and this quantity can be recovered from as little as 1.0 g of tissue. For tissues that contain high concentrations of dsRNA and do not produce viscous extracts, a small column procedure, which still uses only 2.5 g of cellulose (Method 1), works well. The methods have advantages over previously published procedures (4,6,8,12) because they are shorter and therefore quicker, give better recovery, and permit small multiple samples to be processed (3). These are important considerations if the presence of dsRNA is to be used for diagnosis of virus diseases. The method was used to detect the expected dsRNAs in a number of virus-infected plant tissues and also some unexpected low molecular weight components assumed to be dsRNA, such as in TYMV-infected tissue (Figs. 2 and 5). We have been able to isolate such minor replicative forms in other RNA plant virus infections (Fig. 5) using these methods, and the possible significance of this observation has been discussed (7). These observations show that the method may also be useful in the study of the replication of RNA viruses.

Because dsRNA has been detected only in virus-infected plants, the method provides a tool for the study of diseases suspected, but not proved, to be of viral origin because of failure to purify virus particles. Successful isolation of viruses from tissues is dependent on the type of tissue and the stability of the virus, and no single purification method can be used for all viruses. By contrast, a single method for isolating dsRNA from various tissues infected with several different viruses was successful in this study. Preliminary results for several suspected virus diseases from diverse types of

plants indicate that such an approach is possible, and preliminary identification of the casual virus can be made from molecular weight estimates after gel electrophoresis. This approach is being used to detect viruses in rose, grape and cherry tissues; the high content of polysaccharides in these plants makes isolation of nucleic acid difficult by previously applied procedures. In addition, the method already has provided a useful approach for the detection and identification of viruses with dsRNA genomes infecting fungi from which conventional viruslike particles have not yet been purified (1,3).

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