Identification of an Ash Strain of Tobacco Ringspot Virus

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ABSTRACT

Host range tests with a virus isolated from leaves of declining *Fraxinus americana* revealed local and systemic symptoms similar to those caused by ringspot viruses. Properties in crude sap of infected *Vigna sinensis* indicated similarities between the ash isolate and tobacco ringspot virus (TRSV). Three components were separated after sucrose density-gradient centrifugation of the purified virus. The sedimentation velocities of the top, middle, and bottom components were 58, 101, and 127 S, respectively. Infectivity was associated only with the bottom component, and was not enhanced by mixtures with the middle component. Electron micrographs of the bottom component showed isometric particles ca. 26 nm in diam. The purified ash virus reacted positively with TRSV antisera in Ouchterlony agar double-diffusion tests. In reciprocal tests, antisera to the ash virus reacted positively with homologous antigen and TRSV. From these results, the virus isolated from ash was identified as a strain of TRSV.

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Additional key words: tree virus, dieback, component virus.

In a review of virus and viruslike diseases of forest trees, Seliskar (22) listed 53 virus diseases for 34 tree genera. Although graft transmissibility was demonstrated for the majority, few were transmitted to and studied in herbaceous hosts, and none was characterized thoroughly by physicochemical methods. More recently, viruses from forest trees were transmitted to herbaceous plants and identified according to physical properties; e.g., poplar mosaic virus (3), a virus resembling tobacco necrosis virus from *Fraxinus excelsior* (6), black locust mosaic virus (18), elm mottle virus (19), elm mosaic virus (15), and viruses of cacao (5). The causal agents of some of the viruslike diseases in Seliskar's review have since been identified as mycoplasmalike bodies rather than viruses (13). Clearly, viruses infect forest trees, but com-

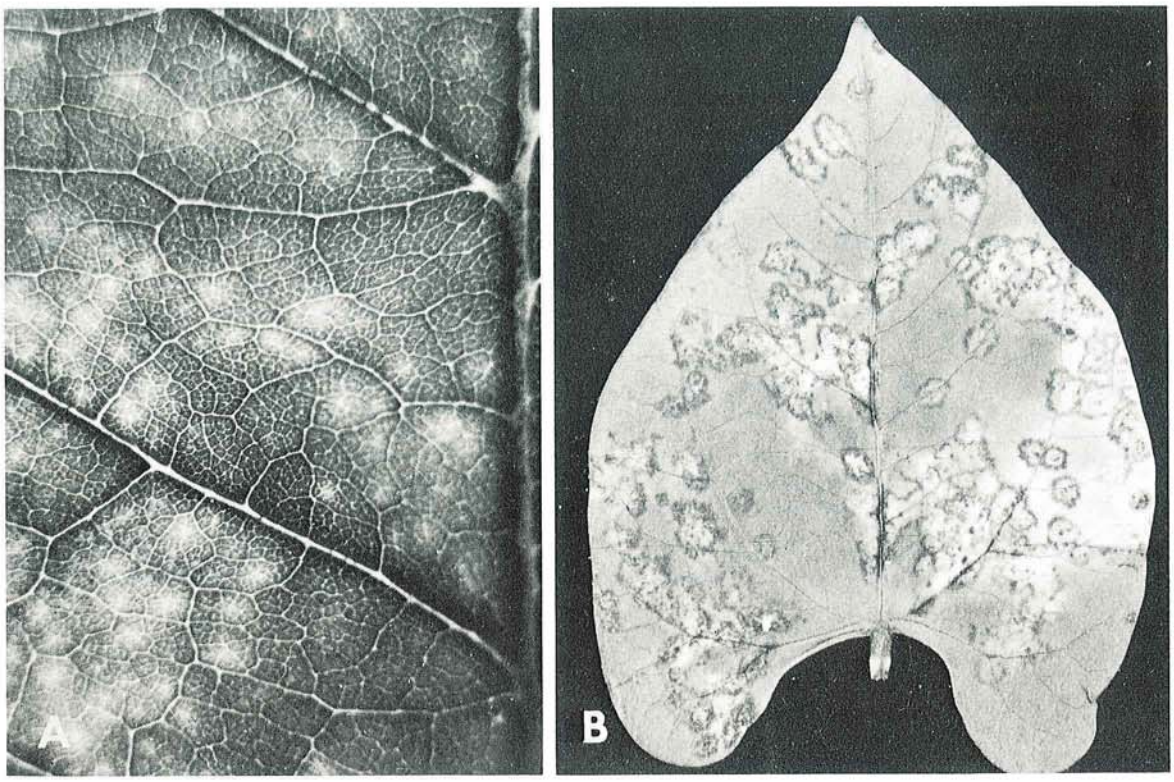

Fig. 1. Foliar symptoms caused by the ash strain of tobacco ringspot virus. **A)** Chlorotic spots on ash (infected by bud grafts). **B)** Reddish brown spots, rings, ringspots, and line patterns on Scotia bean.

paratively little is known about their characteristics, vectors, alternate hosts, and pathogenicity.

Since 1963, symptoms suggestive of virus infection have been observed on leaves of declining white ash (*Fraxinus americana* L.) in southeastern New York. A virus was mechanically transmitted from young ash leaves with faint chlorotic spots and rings (Fig 1-A) to seedlings of herbaceous plants (9). Local and systemic ringspot virus symptoms (single and concentric rings and line patterns) developed on *Phaseolus vulgaris* L. 'Scotia', *Vigna sinensis* (Torner) Savi 'Early Ramshorn', and *Nicotiana tabacum* L. 'Turkish'. The ringspot virus was also graft-transmitted from infected to healthy ash (10). This paper reports the identification and some physical properties of the ringspot virus isolated from ash.

MATERIALS AND METHODS.—Sap expressed from infected primary leaves of Early Ramshorn cowpea was used as virus inoculum. Local-lesion assays were made on Carborundum-dusted, expanded primary leaves of Early Ramshorn cowpea, and inoculated plants were maintained in the greenhouse at 27 C.

Herbaceous host range and symptomatology.—Twenty-two indicator plant species (Table 1) were inoculated by rubbing Carborundum-dusted leaves with infectious sap diluted 1:1 with 0.01 M, pH 7.1, phosphate buffer. Susceptibility to the ash isolate was ascertained by symptom development within 3 weeks after inoculation and by reciprocal inoculations to healthy cowpeas.

Properties in crude sap.—Dilution end point, thermal inactivation point, and longevity in vitro were determined for samples of crude sap using the methods described by Ross (16). Stability at pH 1.0-11.0 was determined for raw sap by adjusting 2-ml samples with 0.1 N NaOH or 0.1 N HCl, incubating at 25 C for 1 hr, readjusting to pH 7.0, and bioassaying on cowpea.

Properties of purified virus.—Preliminary tests with the chloroform-butanol (25) and charcoal (7) methods for purifying tobacco ringspot virus (TRSV) resulted in low virus yields. Consequently, the method of Stace-Smith et al. (24) was used to purify virus from leaves (500-700 g) harvested 5-10 days after inoculation. The purified virus in 0.1 M EDTA buffer was dialyzed overnight against 0.01 M Tris [tris (hydroxymethyl) amino methane] acetate buffer, pH 7.0, containing 0.1 N NaCl before sucrose density-gradient centrifugation.

Sucrose density gradients were prepared by hand layering 2.5, 3.0, 3.0, and 3.0 ml each of solutions containing 100, 200, 300, and 400 mg/ml sucrose in 0.01 M, pH 7.0, phosphate buffer. Virus samples (0.1-0.5 ml) were layered onto the gradients and centrifuged 2 hr at 39,000 rpm in a SW41 rotor of a

TABLE 1. Herbaceous host range of a strain of tobacco ringspot virus isolated from ash and assayed by mechanical inoculation

Family and species	Infection[a] Local	Systemic
Amaranthaceae		
Gomphrena globosa L.	+	0
Apocyanaceae		
Vinca rosea L. 'Pinkie'	+	+
Chenopodiaceae		
Beta vulgaris L.	0	0
Chenopodium quinoa Willd.	0	+
Compositae		
Helianthus annuus L. 'Mammoth Russian'	0	0
Zinnia elegans Jacq.	+	0
Cucurbitaceae		
Citrullus vulgaris Schrad. 'New Hampshire Midget'	0	0
Cucumis sativus L. 'Chicago Pickling'	+	+
Cucurbita maxima Duchesne 'Table Queen'	+	+
Leguminosae		
Crotalaria spectabilis Roth.	+	+
Glycine max Merr.	+	+
Phaseolus vulgaris L. 'Scotia'	+	+
Pisum sativum L. 'Wando'	+	+
Vicia faba L.	+	+
Vigna sinensis (Torner) Savi 'Early Ramshorn'	+	+
Solanaceae		
Datura stramonium L.	+	0
Lycopersicon esculentum Mill. 'Bonny Best'	0	0
Nicotiana tabacum L. 'Turkish'	+	+
N. tabacum L. 'Samsun NN'	+	0
N. glutinosa L.	+	0
Petunia hybrida Vilm. 'Snowstorm'	+	0
Physalis floridana Rydb.	+	+
Solanum tuberosum L. 'Green Mountain'	0	0

[a] + = lesions, ringspots, line patterns, veinal discoloration or clearing, or mottle; 0 = symptomless. Transmission was tested by reciprocal inoculations to cowpea.

Beckman Model L2-65B ultracentrifuge at 5 C. Gradients were pumped through an ISCO UA-2 ultraviolet monitor (ISCO, Lincoln, Nebr.) with an ISCO density-gradient fractionator.

Ultraviolet absorption spectra were made with a Beckman DB spectrophotometer. Analytical ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge at 30,000 rpm using Schlieren optics.

Samples for electron microscopy were placed on carbon-coated Formvar grids, negatively stained with 1% uranyl acetate, and examined in a Zeiss EM9S-2 electron microscope.

For serological assays, purified virus was tested by the Ouchterlony agar double-diffusion method (1) on glass slides in 1% agar containing 0.1 M, pH 7.0, phosphate buffer and 0.85% NaCl. The ash virus was tested against antisera of the following viruses: two strains of TRSV (provided by H. A. Scott, University of Arkansas, Fayetteville, and R. W. Fulton, University of Wisconsin, Madison), *Prunus* necrotic ringspot (provided by W. R. Allen, Canada Department of Agriculture, Vineland Ont.), tomato ringspot, and pea enation mosaic. In addition, crude extracts of infected primary leaves were tested in the laboratory of R. Stace-Smith (Canada Department of Agriculture, Vancouver B.C.) against antisera of the following viruses: sowbane mosaic, strawberry latent ringspot, arabis mosaic, elderberry mosaic, tomato aspermy, cherry leaf roll, elm mosaic, TRSV, raspberry ringspot, tomato blackring, tobacco streak, and tomato ringspot.

Antiserum to the bottom component of purified ash virus was prepared by injecting rabbits at 4-day intervals with one intravenous and two intramuscular injections containing 3 mg each. Serum was taken 2 weeks after the last injection. This antiserum and normal serum were tested against the ash virus, TRSV, tomato ringspot virus, and pea enation mosaic virus.

RESULTS.—*Herbaceous host range and symptomatology.*—The host range of the ash isolate is shown in Table 1. Symptoms on Scotia bean have been described (9) and are illustrated in Fig. 1-B. The local and systemic symptoms caused by the ash isolate were similar to those described for TRSV (23, 26), except that infected Early Ramshorn cowpea reacted somewhat differently. The earliest symptoms on primary leaves were chlorotic spots, which later developed reddish brown margins. Reddish brown spots, rings, and ringspots (0.5-5 mm diam), plus reddish brown discoloration of secondary and tertiary veins, developed in the later stages of infection. Systemically infected and expanding trifoliolate leaves had similar symptoms, plus chlorotic flecks, mottling, and vein clearing. The stem just below the primary leaves, and sometimes the petiole of the first trifoliolate leaflet, often became necrotic and collapsed, which caused wilting and death of the plant.

Properties in crude sap.—The properties in vitro of the virus were as follows: dilution end point, between 10^{-2} and 10^{-3}; thermal inactivation point, between 56 and 59 C; longevity in vitro at 25 C, inactivation between 12 and 24 hr for undiluted sap, for sap diluted 1:1 with 0.02 M, pH 7.0, sodium thioglycollate, and for sap filtered through an ultrafine sintered glass filter; and inactivation by pH, no infection below pH 3.0 or above 10.8.

The ionic strength of the phosphate buffer (0.01, 0.03, 0.05, 0.1, and 0.5 M, adjusted to pH 7.0) added to raw sap had little influence on local-lesion production; water was equally effective as a diluent.

Infectivity was retained up to 4 months when infected cowpea leaves were stored either dried (14) at 4 C or frozen at −10 C. Most infectivity was lost within 1-3 days when raw sap was stored at 4 C or frozen.

Properties of purified virus.—Stability.—To determine the effect of pH and ionic strength on infectivity of the purified virus, 3-ml samples of virus in 0.1 M, pH 7.0, Tris acetate buffer plus 0.1 M NaCl were dialyzed overnight against 0.01-, 0.1-, and 0.2-M solutions of the following buffers: sodium acetate at pH 4.0 and 5.0; sodium phosphate at pH 6.0 and 7.0; and sodium borate at pH 8.0 and 9.0. Bioassay of the dialyzed samples showed similar infectivity for the samples exposed to pH 5.0-8.0 at all molarities. The results at pH 4.0 and 9.0 were variable, but some infectivity was retained at all three molarities.

Purified virus stored at 4 C in 0.01 M, pH 7.0, phosphate buffer containing 0.1 M NaCl retained infectivity for at least 3 weeks.

Virus components.—The ultraviolet absorbancy profile following sucrose density-gradient centrifugation of the purified virus is shown in Fig. 2. A large band accounting for about 90% of the ultraviolet absorbancy was designated bottom component. It had a typical nucleoprotein absorbancy spectrum with a 280:260 nm ratio of 0.58, and infectivity was closely associated with this peak when assayed either directly or adjusted to 0.025 OD_{260} nm/ml prior to bioassay. Electron micrographs of bottom component contained isometric particles ca. 26 nm diam (Fig. 3-B). Sedimenting closely behind bottom component was a pair of incompletely separated ultraviolet absorbing bands which were collectively designated as middle component. They had a nucleoprotein spectrum and contained isometric particles identical in appearance to those in bottom component. Infectivity was not associated with middle component. A top component fraction sedimented as a broad band. Electron micrographs of this fraction (Fig. 3-A) contained particles about 29 nm in diam which were both penetrated and unpenetrated by uranyl acetate, plus many small spherical particles typical of ferritin (4). The ultraviolet absorption spectrum of this band was also typical of ferritin.

Sedimentation velocities, as determined by analytical ultracentrifugation, for bottom, middle, and top components were $S_{20,w}$ = 127, 101, and 58, respectively.

To further test infectivity of the bottom (B), middle (M), and top (T) components, fractions comprising each of the three ultraviolet-absorbing bands were pooled, then pelleted by high-speed centrifugation and resuspended in 0.01 M, pH 7.0, phosphate buffer. The pooled fractions of B, M, and T were then centrifuged on sucrose density-gradient columns and fractionated into 0.5-ml fractions (Fig. 4). Pooled T contained a broad top component peak and had no infectivity. Pooled M had a double peak corresponding to middle component, plus a small peak corresponding to bottom component. Infectivity was associated only with the bottom component peak. Pooled B contained no middle or top component, and infectivity was associated with the ultraviolet absorbancy of the bottom component peak and two small peaks (aggregates) sedimenting faster than bottom component.

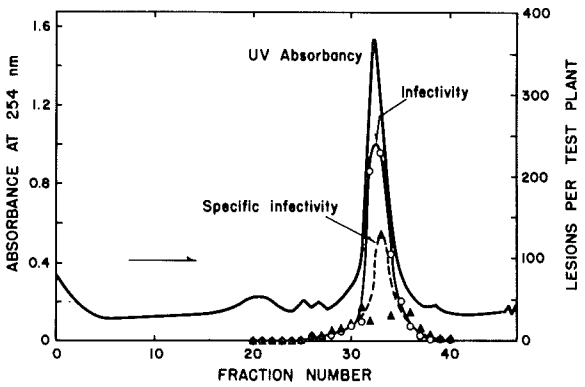

Fig. 2. Optical density profile, infectivity, and specific infectivity following centrifugation of the ash strain of tobacco ringspot virus in a 100-400 mg/ml sucrose density gradient for 2 hr at 39,000 rpm in a Beckman SW41 rotor at 5 C.

Test for activation of bottom component by middle component.—Experiments were conducted to determine whether mixtures of middle and bottom components were more infective than the bottom component alone. Samples of the two component fractions were adjusted to 0.025 OD_{260} nm/ml, singly diluted 1:1 with 0.01 M, pH 7.0, phosphate buffer or in mixtures of bottom plus middle component, and bioassayed by the opposite half-leaf method. The results (Table 2) indicate that only bottom component was infective, and that middle component did not activate bottom component. The slight infectivity in middle component probably resulted from contaminating bottom component in the sample (Fig. 4).

Serology.—Figure 5 shows precipitin bands between wells containing the ash virus and its homologous antiserum and two preparations of antisera to TRSV. When the ash antigen was tested against its homologous antiserum in wells alternating with TRSV antisera, a single merging precipitin band developed indicating close serological relationship. The ash virus did not react with normal serum or with antisera to *Prunus* necrotic ringspot, pea enation mosaic, or tomato ringspot viruses. In reciprocal tests, antiserum to the ash virus, with a titer of 1:512, reacted only with its homologous antigen and with TRSV.

The serological relationship between the ash virus and TRSV was confirmed by R. Stace-Smith (*personal communication*). A crude extract of cowpea primary leaves infected by the ash isolate reacted only with antiserum to TRSV.

DISCUSSION.—On the basis of host range, properties in crude sap, properties of purified virus, and serological reactions, the ringspot virus isolated from ash leaves is identified as the ash strain of tobacco

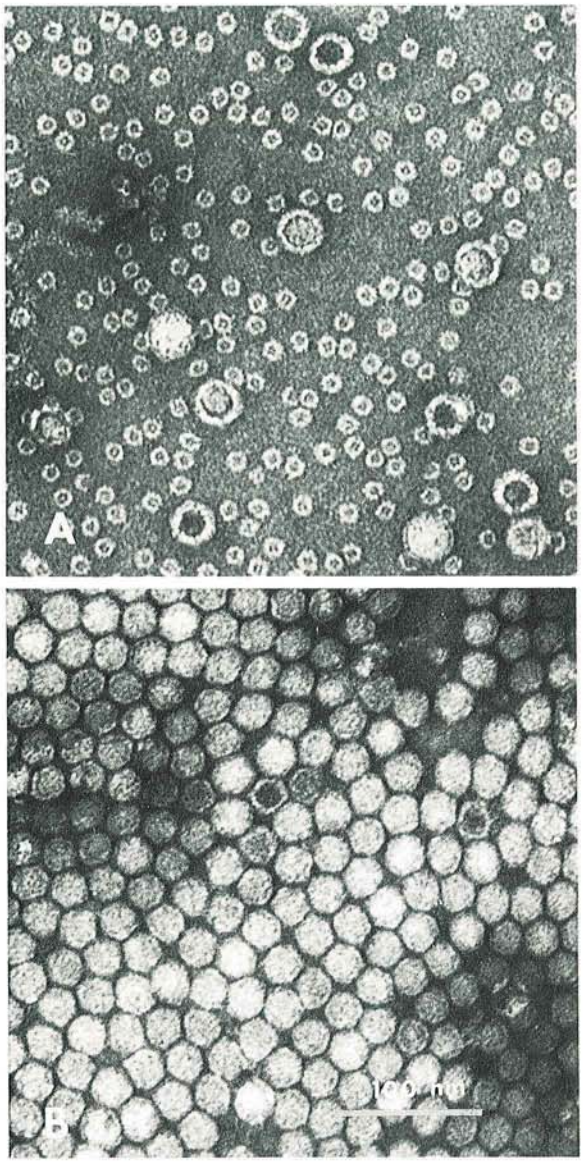

Fig. 3. Electron micrographs of the ash strain of tobacco ringspot virus purified by sucrose density-gradient centrifugation. Top component fraction **A)** contains apparently empty capsids (large particles) and ferritin particles (small particles), and bottom component fraction **B)** contains only virions negatively stained with uranyl acetate.

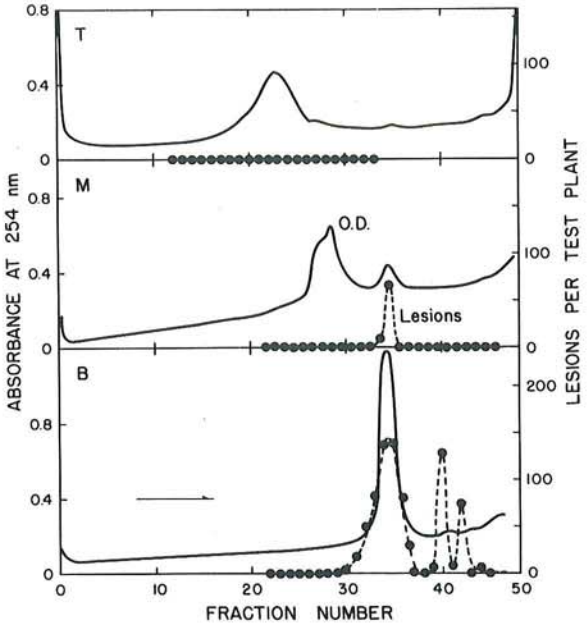

Fig. 4. Optical density profile and infectivity of top (T), middle (M), and bottom (B) components of the ash strain of tobacco ringspot virus purified by two sucrose density-gradient centrifugation cycles for 2 hr at 39,000 rpm in a Beckman SW41 rotor at 5 C.

ringspot virus (TRSV-A). Infection by TRSV-A caused symptoms on herbaceous indicator plants that were typical of those produced by TRSV (23, 26). The properties in crude sap were similar to those described for TRSV (23), except that in longevity tests, TRSV-A lost infectivity in 12-24 hr as compared to 3-4 days for TRSV.

Separation of purified TRSV-A by sucrose density-gradient centrifugation into top, middle, and bottom components (sedimentation velocities of 58, 101, and 127 S, respectively) agrees with reports for TRSV. Stace-Smith et al. (24) reported 53, 94, and 128 S; Schneider & Diener (20) reported 53, 91, and 126 S for top, middle, and bottom components, respectively. Steere (25) measured a minor and a major component for TRSV, with sedimentation velocities of 89 and 116 S, respectively.

Schneider & Diener (20) reported a broad peak for the top component of TRSV. The top component of TRSV-A also appeared as a broad band, and was contaminated with ferritinlike particles (4). Ferritin is an iron-protein complex which is known to accumulate in high concentrations in legumes (12, 21). For this reason, the antiserum to TRSV-A was prepared from the bottom component purified by sucrose density-gradient centrifugation.

The middle component of TRSV-A differs from previously reported strains of TRSV in that its quantity relative to that of bottom component is quite small, and because it contains two subpopulations. No attempt was made to fractionate and purify the subpopulations.

Purified virus preparations containing two or more sedimenting components are sometimes more infective when more than one component is present in the inoculation mixture. This type of activation was first reported by Wood & Bancroft (27) for the bean pod mottle and cowpea mosaic viruses, and is now considered to be a general phenomenon within the squash mosaic group of viruses (2). Our results do not indicate an interaction between the middle and bottom components of TRSV-A. Apparent high-

TABLE 2. Infectivity on 28 paired half-leaves of cowpea inoculated with middle (M), bottom (B), or mixtures of M and B components of ash virus, each adjusted to 0.025 OD_{260} nm/ml

Experiment	Average no. lesions for paired preparations		
	M vs. B	M vs. B + M	B + M vs. B
1	4.4/42	2.4/31.4	59.7/65.2
2	1.9/5.4	0.7/3.9	4.5/5.1
3	4.9/90.6	4.9/71.7	51.1/51.4
4	0.1/4.8	0.1/3.6	7.3/5.2

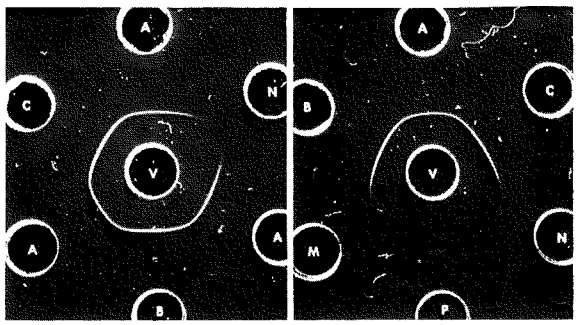

Fig. 5. Ouchterlony double-diffusion reactions of (V) the ash strain of tobacco ringspot virus (TRSV-A); (A) antiserum to TRSV-A; (B, C) antisera to two strains of tobacco ringspot virus; (M) antiserum to tomato ringspot virus; (P) antiserum to pea enation mosaic virus; and normal sera (N) of the rabbit used for the preparation of antiserum to TRSV-A.

specific activity was associated with the small peaks sedimenting faster than bottom component (Fig. 4). If these are, in fact, dimer and trimer peaks, the data suggest enhanced infectivity. These fast peaks were not replicated in our experiments; therefore, there are insufficient data to postulate that this represents activation.

On the basis of deZeeuw's host list for TRSV (8), we believe this to be the first report of the natural occurrence of a strain of TRSV in a major forest tree species. Ash seedlings have been infected with TRSV-A by mechanical inoculation (Hibben, *unpublished data*), and a nematode vector has been identified (11), but the distribution of this virus in the field and its role in the etiology of ash dieback (17) are unknown. The wide host range of TRSV in both herbaceous and woody plants and the recognition of its nematode vector suggest that strains of this virus may infect other forest tree species.

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