Purification and Composition of Potato Virus Y

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ABSTRACT

Potato virus Y (PVY) was purified from systemically infected leaves of *Nicotiana tabacum* 'Haranova'. Of several procedures investigated for preliminary clarification of expressed sap, the most effective was alcohol or heat precipitation prior to differential centrifugation. A high molarity buffer to suspend the first high-speed pellet reduced to a minimum the aggregation of virus particles. The virus was further purified by sucrose-gradient centrifugation, where PVY sedimented as a single opalescent zone with a sedimentation coefficient of 150 S. The yield of purified virus was 3-4 mg/kg of leaves. The ultraviolet spectrum of the purified virus was typical of other tubular viruses, with absorption maximal at 260 nanometers (nm) and minimal at 247 nm. The purified virus had a phosphorus content of 0.49%, indicating a nucleic acid content of 5.4%. The amino-acid composition of the virus was similar to that of tobacco etch virus protein, another virus in the PVY group. The virus moved toward the anode in agar gel electrophoresis at pH 7.4. Phytopathology 60:1785-1789.

In a classification of elongated plant viruses based upon gross morphology and serology (1), one group is designated the "Potato Virus Y" group. Twenty viruses are included in this group, ranging in length from 730 nm to 790 nm. All are flexible particles having a diam of about 15 nm, and at least some show distant serological relationships. Another characteristic in common is that the viruses occur in relatively low concn in infected sap. Their low concn and tendency to aggregate during purification has meant that few viruses of the group have been obtained in sufficient quantity and purity for physical or chemical study.

Wetter (12), in a serological study of elongated plant viruses, purified PVY by using ether and carbon tetrachloride for preliminary clarification followed by differential and rate-zonal density-gradient centrifugation. Used as an antigen for intravenous and intramuscular injection, the product gave an antiserum of high titer, but other properties of the virus were not investigated. Delgado-Sanchez & Grogan (4) used chloroform for preliminary clarification, and differential and sucrose-gradient centrifugation for further purification. Their preparation sedimented as a single band in the gradient, with a sedimentation constant of 154 S.

In our study of PVY, we confirmed that the virus could be purified using ether, carbon tetrachloride, or chloroform for initial clarification and differential centrifugation, and sucrose gradient centrifugation for further purification. As previous workers have found, the yield of purified virus was extremely low. We therefore investigated other purification procedures, and eventually developed a schedule that yielded sufficient virus for physical and chemical determinations.

Since tobacco etch virus (TEV) is the only virus in the PVY group whose amino-acid composition has been determined, we compared results of Damirdagh & Shepherd (3) with our amino-acid analyses of PVY. The purification procedure, with physical and chemical characteristics of the virus, form the subject of this paper.

Materials and Methods.—Tobacco seedlings (*Nicotiana tabacum* L. 'Haranova') were inoculated with a culture of PVY obtained from Frank Manzer, University of Maine. The plants used for virus production were grown either in a greenhouse under natural light conditions and at temp ranging from 20-23 C, or in a growth room under fluorescent lights, averaging 1,000 ft-c for a 16-hr day at temp of 23-25 C. Plants were inoculated at the 3-5 leaf stage and grown in 10-inch pots of John Innes No. 2 soil mixture. About 4 weeks after inoculation, when the leaves showed a distinctive mottle, the large (30-40 cm long) basal leaves were harvested. Further batches of large mature leaves were harvested over the succeeding 4-week period.

Several methods of virus purification were investigated, and the significant findings are presented later. The procedure that was eventually found satisfactory and used to obtain the virus preparations was as follows: (i) Sprinkle sodium diethyldithiocarbamate (DIECA) over the leaves at 5 g/kg, and crush in an Erweka Type KU1 fruit press. Extract the juice through a cheesecloth and clarify by low speed centrifugation at 14,000 *g* for 20 min. (ii) Heat the supernatant fluid to 41 C for 2 min or add 95% alcohol, 20 ml/100 ml sap. Clarify by low-speed centrifugation at 14,000 *g* for 20 min. (iii) Centrifuge the clarified extract at 70,000 *g* for 2 hr. Cover each pellet with 2 ml of phosphate buffer, 0.5 M, pH 7.0, and stand overnight at room temp. (iv) Suspend the pellets with a glass rod, combine, and centrifuge at 9,000 *g* for 20 min. (v) Concentrate the supernatant fluid (about 10-fold) by making a slurry with Sephadex G-25 Coarse and applying gentle suction through a fritted glass filter to obtain the void volume. (vi) Layer 2-3 ml on a 6-30% sucrose gradient prepared in 0.5 M phosphate buffer and centrifuge at 50,000 *g* for 2 hr in a Spinco SW25.1 rotor. The method used to prepare the gradients has been described (10). (vii) Extract the opalescent zone with a hypodermic syringe and store at 4 C. If prolonged storage is anticipated, add sodium azide at 1:10,000.

The purification procedure was modified somewhat in preparing virus that was used for phosphorus analysis. We used 0.5 M phosphate buffer for suspending the pellets (step 4), but used 0.01 M Tris[tris(hydroxymethyl) aminomethane]-HCl, pH 7.2, buffer for

the sucrose gradient and a higher sucrose concn (12-40%). The virus zone from the sucrose-gradient tubes was exhaustively dialyzed against Tris-HCl to remove any traces of phosphate.

Virus loss sustained in clarification procedures was assessed by determining the dilution end point of unconcentrated sap in tube precipitin serological tests. Preparations that had been concd by centrifugation were examined in the Schlieren optics of the analytical ultracentrifuge. This provided information on the virus yield resulting from the procedure, and indicated the degree of purity.

Sedimentation coefficients were determined over a range of virus concn and in several buffer solutions. The Spinco Model E analytical ultracentrifuge was run at the speed setting of 31,410 at 20 C, and photographs were taken at 4-min intervals. Sedimentation coefficients were calculated using the graphical method of Markham (6).

Separation and recovery of the nucleic acid from the virus was unsuccessful by two methods: addition of 1 N HCl (11) and 6 M guanidine (8).

The amino-acid composition was determined in 10 preparations. Most analyses were made on the virus protein which was isolated from nucleic acid by dialysis of the virus against 1 N NaOH for 2 days at 4 C. The protein remained soluble after dialysis against distilled water, and aliquots were pipetted into two tubes. After the addition of an equal volume of 12 N HCl, the tubes were evacuated, sealed, and hydrolyzed in an oven at 107 C for 24 and 72 hr. After hydrolysis, the excess HCl was removed in a flash evaporator and the samples were dissolved in 5.0 ml of citrate buffer, pH 2.2. Aliquots of 1 ml were analyzed with a Spinco amino-acid analyzer. Cysteine and methionine determinations were made on two separate preparations of virus oxidized with performic acid before hydrolysis for 24 hr. Aliquots of another virus preparation were used for the analysis of tryptophan content (9), and the amount of virus protein in this preparation was determined by amino-acid analysis.

Inorganic phosphorus was determined by the method of Chen et al. (2) from samples of whole virus heated at 190 to 200 C for 15 min with 70% perchloric acid. The virus had previously been dialyzed at 4 C against Tris-HCl buffer for 2 days. The amount of virus protein in the preparation was determined by amino-acid analysis.

Agar electrophoresis was carried out in 9 × 9 cm plastic trays. The agar was 2 mm thick and consisted of 0.7% Ionagar in 0.02 M, pH 7.4 Tris-HCl buffer. The virus preparations were placed in 3-mm wells cut with a cork borer, and current was applied at 200 v for 4 hr. The viruses were located by staining with 1% nigrosine solution in 10% acetic acid and destaining with 2% acetic acid.

RESULTS.—*Virus purification.*—Yield from plants that were grown under controlled conditions was relatively uniform and predictable, but variable results were obtained from plants grown in the greenhouse. In general, yields were satisfactory during the winter and early spring, but unsatisfactory during the summer. Plants grown for a long period without supplementary nutrients produced yellow leaves with a low virus content. Virus content of mature lower leaves was similar to that of immature upper leaves, and remained fairly constant over the 4-week harvesting period. Harvesting only the mature leaves thus maximized the total yield of infected tissue per plant.

The best results were obtained without the addition of buffer in the initial extraction, adding only dry powdered DIECA at 5 g/kg of leaves before they were crushed. Sap from such extractions, when clarified by low-speed centrifugation, usually showed a visible precipitate in the tube precipitin serological test at a dilution of 1:32 but not at 1:64. When leaves were frozen overnight, the titer of the clarified sap was usually reduced to about 1:8. The inclusion of DIECA did not increase the virus titer, but did reduce the brown coloration in the final steps of purification.

Sap that was extracted, clarified by low-speed centrifugation, and subjected to high-speed centrifugation yielded large green pellets. Recovery of purified virus from such pellets was difficult; hence, a number of preliminary clarification procedures were investigated. Adjusting the sap to pH 5.5 or pH 5.0 with dilute hydrochloric acid incurred extensive virus loss. Adding 20 ml of 95% ethyl alcohol to 100 ml of sap resulted in only a slight virus loss and a cleaner preparation (Fig. 1-A). Heating the sap to 41 C caused coagulation of some healthy constituents that could be sedimented by low-speed centrifugation without appreciable loss of virus, but when the sap was heated above 41 C for more than a few min, a high proportion of the virus particles were coprecipitated with the healthy plant constituents. Sap that had been heated at 41 C and clarified by low-speed centrifugation still yielded large greenish pellets when concd by high speed centrifugation, but more virus was recovered from these pellets than from sap that had not been heat-treated (Fig. 1-B).

It was difficult to recover the virus from high-speed centrifuge pellets. For good recovery, it was essential to suspend the pellet in a high molarity buffer such as 0.5 M phosphate pH 7.0. In a low molarity buffer such as 0.01 M phosphate, pH 7.0, a large proportion of the virus particles either aggregated or adhered to healthy constituents and was lost in low-speed centrifugation.

When the suspended pellets were clarified by low-speed centrifugation and the supernatant solution was examined in the analytical ultracentrifuge, there was a small virus peak and a slow-moving peak thought to be Fraction 1 protein (Fig. 1-C). The virus was concd further by another high-speed centrifugation or by making a slurry with Sephadex G25 Coarse and extracting the void volume. Both methods were equally effective (Fig. 1-D). Centrifugation reduced the relative concn of the contaminating Fraction 1 protein, but extraction through Sephadex was more rapid and was the preferred method. The virus solution at this stage of purification was a light brown, and contained a trace of material that sedimented faster than the virus, in addition to the Fraction 1 protein. Virus solutions that

showed a clear peak in the Schlieren optics gave a distinct light-scattering zone after centrifugation on a sucrose gradient. The yield of purified virus was 3-4 mg/kg of leaves.

Ultraviolet absorption.—The absorption spectrum of a solution of purified PVY (Fig. 2) was typical of tubular plant viruses. The spectra of five separate preparations showed min and max absorption of 247 nm and 260 nm, respectively. The 260:280 ratio was 1.21 ± 0.04, and 260:247 ratio was 1.11 ± 0.02.

Sedimentation rate.—The S values for eight solutions of PVY varied from 138 to 148, and were dependent upon the virus concn. A solution containing 1.0 mg/ml in 0.04 M phosphate buffer containing 0.06 M sodium chloride sedimented at 138 S. Another solution in the same buffer but containing only 0.2 mg/ml of virus sedimented at 147 S. Still another solution containing 0.2 mg/ml in 0.01 M phosphate buffer without sodium chloride sedimented at 148 S. Extrapolated to infinite dilution, the sedimentation coefficient ($s_{20\,w}$)

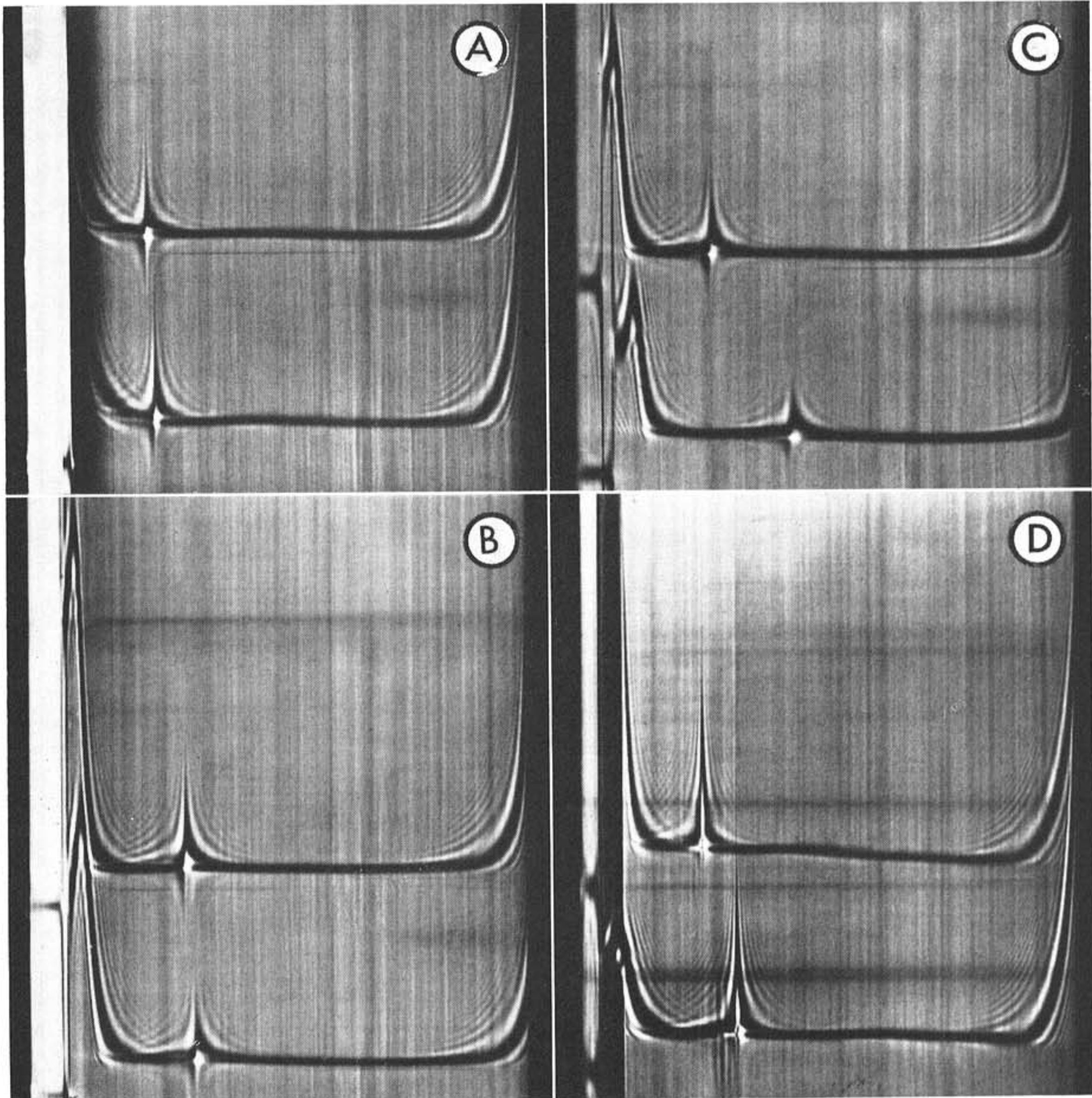


Fig. 1. Upper (U) and lower (L) schlieren patterns of two-cell analytical ultracentrifuge runs comparing potato virus Y purification treatments. **A)** Alcohol added (20 ml of 95%/100 ml sap) prior to high speed-centrifugation (U) and water added as a control (L). **B)** Sap heated to 41 C for 2 min prior to high-speed centrifugation (U) and control sap held at room temp (L). **C)** High-speed pellet suspended in 0.5 M phosphate buffer, pH 7.0 (U), and 0.01 M phosphate buffer, pH 7.0 (L). **D)** High-speed pellet concd by extracting from a slurry with Sephadex G25 Coarse (U) centrifuging at 85,000 *g* for 90 min (L). Photographs were taken between 10 and 15 min after reaching a speed of 35,600 rpm. Bar angle was 60 degrees.

for PVY was 150 S, a value in good agreement with the 154 S determined by Delgado-Sanchez & Grogan (4).

Amino-acid analyses.—Table 1 shows the results of amino-acid analyses of virus protein. The values for serine and threonine were obtained by extrapolation to zero hydrolysis time. Based on these values, the losses of serine and threonine were 91% and 95%, respectively. Isoleucine and valine yielded greater recoveries after 72-hr hydrolysis, which agrees with the greater stability of peptide bonds involving these amino acids. Variations in yield of the other amino acids were within the accuracy of the method.

The cysteine and methionine content were determined by analyses of protein oxidized with performic acid. The values for cysteic acid and methionine sulfone were 400% and 140%, respectively, greater than the quantities of cysteine and methionine recovered after hydrolysis of the protein.

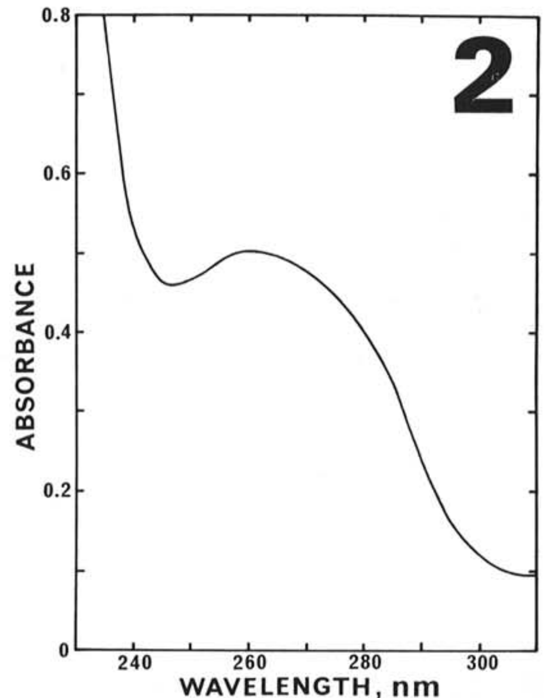
Since tryptophan is destroyed during acid hydrolysis, it was determined by the *p*-aminobenzaldehyde method (9). Spectrophotometric scanning and *N*-bromosuccinimide were not used because the protein was not free of nucleic acid. The analyses of virus protein (Table 1) agreed well with analyses of six whole virus preparations (not shown). The yields of glycine and alanine, however, were usually greater than those shown in Table 1, probably because of the degradation of nucleic acid (7).

The molar percentages of amino acids in PVY protein were multiplied by 1.94 to yield relative molar ratios for comparison with tobacco etch virus. The quantities of PVY obtained were not sufficient to determine the number of amino acids in the protein subunit by the methods used with TEV. Although there is no proof that PVY and TEV protein are the same size, their compositions are similar, and differences would represent only 15 amino-acid exchanges. The greatest difference was in isoleucine. Three analyses of two different virus preparations gave values of 0.49 ± 0.02% phosphorus by wt. Damirdagh & Shepherd (3) calculated the nucleic acid of TEV to be 9.13% phosphorus by wt. Using this value, PVY would contain 5.4% nucleic acid.

The extinction coefficient was calculated from amino acid analyses and OD measurements of a virus preparation. A value of 2.86 at 260 nm for 1 mg/ml of virus was calculated on the assumption that the virus contained 5% RNA.

Electrophoretic mobility.—The electrophoresis of PVY in agar at pH 7.4 (Fig. 3) showed that the virus moves to the anode. The electrophoretic mobility of PVY is greater than that of two other rod-shaped viruses, potato virus X (PVX) and potato virus M (PVM). The movement of PVX toward the cathode was due to electrophoretic endosmosis in the agar gel, since Loring (5) found that PVX was isoelectric at pH 4 to 5 in moving boundary electrophoresis. Turnip crinkle virus (TCV) was included in this test to demonstrate a uniform electrical field in the agar. Comparison of movement of PVY with the spherical virus, TCV, will not be made, since the agar matrix would tend to restrict movement of rod-shaped viruses.

Discussion.—Potato virus Y occurred in such low concn that it did not show as a peak when crude sap was examined in the schlieren optics of the analytical ultracentrifuge; however, the virus concn was enough to obtain a serological reaction with approx 40-fold dilution; hence, serological determinations of dilution end points may be useful in assessing various techniques for expressing and clarifying sap. Alternatively, comparable samples may be concd by high-speed cen-



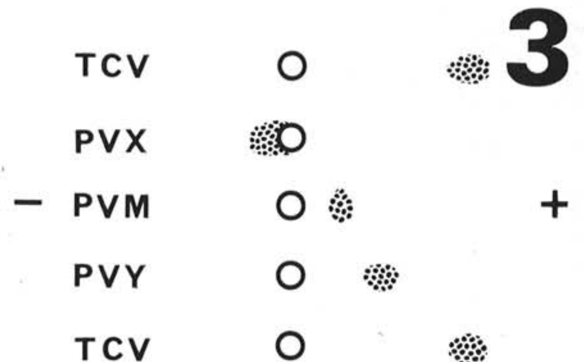


Fig. 2-3. 2) Ultraviolet absorption spectrum of a purified preparation of potato virus Y. The approximate concn of virus is 0.18 mg/ml. **3)** Drawing of stained agar electropherogram showing positions of turnip crinkle virus (TCV), potato virus X (PVX), potato virus M (PVM), and potato virus Y (PVY) after electrophoresis at pH 7.4 for 4 hr at 200 v.

TABLE 1. Amino acid composition of potato virus Y protein

Amino acid	Nanomoles of amino acids recovered			Molar percentage	Relative molar ratio[f]	Relative molar ratio for TEV[g]
	24-Hr hydrolysis[a]	72-Hr hydrolysis[a]	Avg value			
Alanine	508	520	514	8.40	16	19
Arginine	359	362	361	5.90	11	13
Aspartic acid	701	691	696	11.37	22	25
Cysteine[b]	22		22	0.36	1	1
Glutamic acid	734	724	729	11.91	23	23
Glycine	412	418	415	6.78	13	13
Histidine	144	140	142	2.32	4	6
Isoleucine[c]	329	367	367	6.00	12	5
Leucine[c]	301	307	307	5.02	10	13
Lysine	387	409	398	6.50	13	10
Methionine[b]	260		260	4.25	8	10
Phenylalanine	149	153	151	2.47	5	5
Proline	361	333	347	5.67	11	8
Serine[d]	284	228	312	5.10	10	9
Threonine[d]	400	358	422	6.90	13	13
Tryptophan[e]			73	1.19	2	2
Tyrosine	210	200	205	3.35	6	7
Valine[c]	372	398	398	6.50	13	12
		Total	6,119	100.0	193	194

[a] Average of two analyses.
[b] Determined from performic acid-oxidized protein.
[c] Only 72-hr hydrolysis analyses included in average.
[d] Extrapolated to zero hydrolysis time.
[e] Determined by the colorimetric analyses of Spies & Chambers (9).
[f] Molar percentages were multiplied by 1.94 for comparison with tobacco etch virus protein.
[g] Tobacco etch virus, from Damirdagh & Shepherd (3).

trifugation, and the suspended pellets compared in the analytical ultracentrifuge. Concentrating expressed sap about 10-fold yields material that is suitable for comparative analysis in the analytical ultracentrifuge. In any event, the use of serology in assessing the initial steps of purification is optional and, in the subsequent steps in purification, the analytical ultracentrifuge is an invaluable tool. Not only do photographs show the absolute virus yield, but the homogeneity of the virus peak indicates whether it will sediment as a diffuse or a sharp zone upon sucrose-gradient centrifugation. Also, nonviral contaminants that may interfere with purification on a sucrose gradient are revealed by examination in the schlieren optics.

Another technique for assessing virus loss during PVY purification is rub-inoculation of the local lesion host *Chenopodium quinoa* (4). This technique has the same limitation as serology, in that it gives no indication of virus homogeneity or of nonviral contaminants. For this reason, and because more than a week is required to develop local lesions, we did not use local lesion indicators in our experiments.

The phosphorus content of PVY (0.49%) is similar to the 0.47% value determined for TEV. The value of 5.4% nucleic acid for PVY calculated from the phosphorus content assumed that the nucleotide composition of PVY was the same as that of TEV. The difficulty encountered with PVY in separating RNA from protein precludes the possibility of an exact comparison of percentage RNA in PVY and TEV, but the phosphorus analyses are close.

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