Preservation of Plant Virus Antigens by Freeze-Drying

D. E. Purcifull, S. R. Christie, and D. L. Batchelor

Plant Virus Laboratory, Department of Plant Pathology, University of Florida, Gainesville 32611.
Florida Agricultural Experiment Stations Journal Series Paper No. 5849.
Supported in part by Grant No. GB-32093 from the National Science Foundation.
The authors gratefully acknowledge the technical assistance of Eugene Crawford.
Accepted for publication 13 May 1975.

ABSTRACT

Serological activities of 13 plant viruses were retained for extended periods in freeze-dried crude extracts from infected plants. Prior to use in immunodiffusion tests, the extracts containing rod-shaped viruses were suspended either in 1.5% sodium dodecyl sulfate or other degrading agents, and the isometric viruses were suspended in water. Nine of the viruses were antigenically active after storage for at least 1 year, and the others were active for the longest storage period tested (at least 1 month). The viruses used included six in the potato Y group, three in the potato X group, tobacco mosaic virus, and three isometric viruses (brome mosaic virus and two strains of southern bean mosaic virus). Immunochemical specificities of the freeze-dried antigens of a particular virus were comparable to those of the corresponding virus in freshly prepared extracts, based on tests with pepper mottle, potato X, potato Y, southern bean mosaic, and tobacco etch viruses. The freeze-drying technique has proved convenient for maintaining a collection of plant virus antigens for immunodiffusion tests.

Phytopathology 65:1202-1205

Additional key word: lyophilization.

Purified virus preparations or freshly prepared plant extracts have been commonly used as antigens for serological tests with plant viruses. Other types of preparations used in immunodiffusion tests include filter paper disks containing lyophilized barley stripe mosaic virus antigens (7), antigens extracted from dehydrated tissues (3, 10, 13) and antigens extracted from leaf tissue stored in sodium azide (5). The general success of freeze-drying as a means of preserving plant virus infectivity (e.g., 8, 9) suggested that this method might be applicable to the preservation of antigenic activity, but specific documentation of this point is lacking for most viruses. The present study reports the successful use of freeze-drying for preserving the serological activity of three isometric viruses, and for preserving serological activity of antigens obtained by sodium dodecyl sulfate (SDS) treatment of ten anisometric viruses. The benefits of maintaining a collection of freeze-dried virus antigens are discussed.

MATERIALS AND METHODS.—The potato virus X (PVX) isolate used was the mottle strain from J. F. Shepard. Southern bean mosaic virus (SBMV) was obtained from J. G. McDonald, and the cowpea strain of SBMV (designated SBMV-C) was provided by J. Wakeman. The tobacco etch virus (TEV) used for most studies was the American Type Culture Collection (ATCC) No. PV-69. A severe TEV isolate from G. V. Gooding, Jr., and a TEV isolate (designated Oxnard isolate) from P. Smith were also used. The bidens mottle virus used was ATCC No. PV-165, and the pepper mottle virus (PeMV) was the isolate recently described (12). Brome mosaic virus (BMV) was obtained from D. Pring and tobacco mosaic virus was obtained from D. A. Roberts. Lettuce mosaic virus was isolated from lettuce seedlings infected by seed transmission, and the turnip mosaic virus was from naturally infected turnip. Potato virus Y (PVY) was the isolate from North Carolina used previously (10). The papaya mosaic and clover yellow mosaic virus isolates were also used in a prior study (1).

The antisera to the rod-shaped viruses were prepared by injecting rabbits intramuscularly with antigen preparations emulsified 1:1 (v/v) with Freund's incomplete or complete adjuvant (Difco). Prior to injection, the purified virus preparations were either untreated (bidens mottle, lettuce mosaic, PeMV, PVY, tobacco mosaic, and turnip mosaic viruses) or denatured in SDS (1) (clover yellow mosaic, papaya mosaic, and PVX). TEV was either untreated (serum used in Fig. 1-B) or treated with ethanolamine (serum used in Fig. 1-E) prior to injection.

Antisera to the southern bean mosaic viruses were prepared by injecting purified virus into rabbits (1). The antiserum to BMV (PV-AS No. 61) was obtained from the ATCC.

All antisera were used undiluted except for the BMV antiserum, which was diluted (1:4, v/v) with 0.05 M Tris, pH 7.2, containing 0.85% NaCl.

For freeze-drying of extracts from healthy or virus-infected plants, freshly harvested tissues were triturated in water (1 g/ml) with a mortar and pestle, the pulp was expressed through cheesecloth and divided into two equal samples. Deionized water was added to one sample (1:1, v/v) and 3% SDS in H_2O to the other sample (1:1, v/v). Alternatively, SDS-treated extracts were prepared by grinding tissue in water (g/ml), to which was added 3% SDS (1 ml/g tissue) before the pulp was expressed through cheesecloth (11). Samples (usually 2 ml) were pipetted into Virtis vac-vials, partially stoppered with Virtis split rubber stoppers, and either placed directly on the temperature-controlled shelf of a Thermovac Model FDC-32-SR-P3-MOD freeze-drier at −40 C, or pre-frozen at −20 C in a freezer for a few days. After the samples on the shelf were frozen, vacuum was applied. After a pressure of 250 μm of Hg was reached, the shelf temperature was raised to 22 C. The samples were freeze-dried overnight (or longer) and the vials were sealed under vacuum with the stoppering diaphragm. The SDS-treated samples were then stored at room temperature and reconstituted with water prior to use. Samples extracted in water were routinely stored in a freezer and suspended before use with one of the following, depending on the purpose of the test: water, 1.5% SDS, 2.5% pyrrolidine

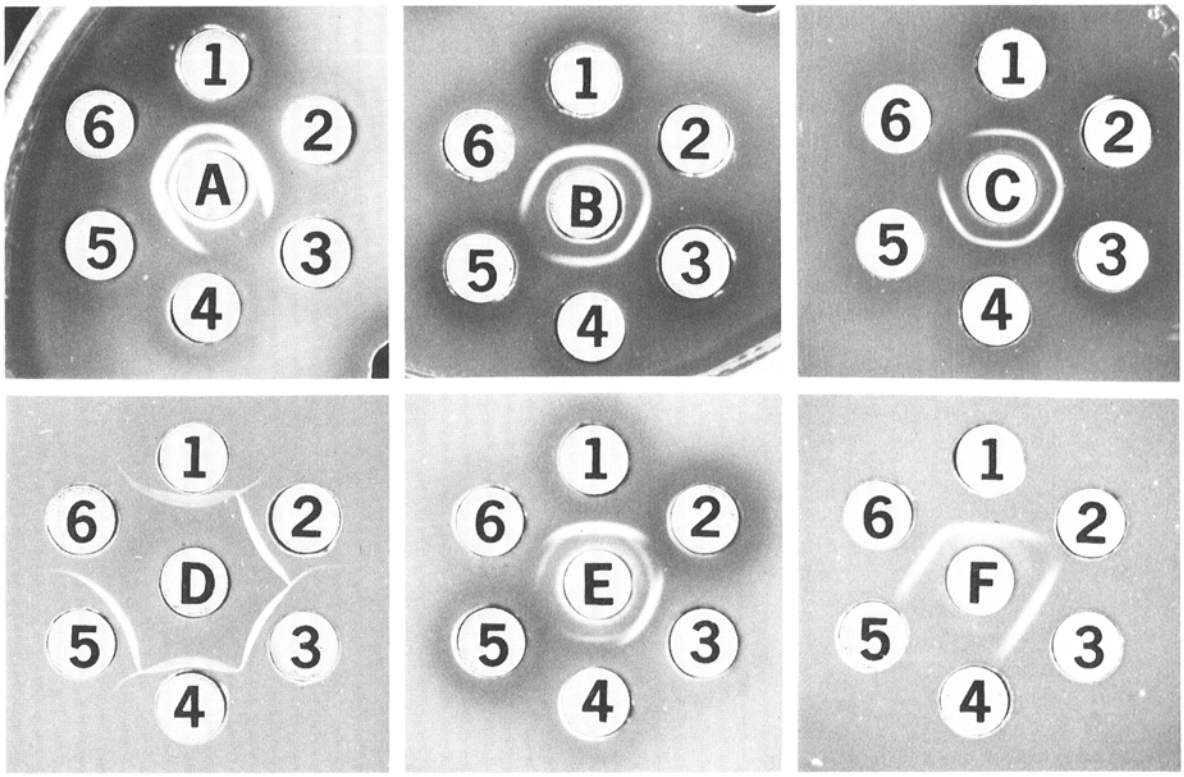

Fig. 1-(A to F). Photographs of immunodiffusion tests illustrating immunoreactivities of freeze-dried reconstituted extracts from virus-infected plants. The center wells were charged with antisera and the peripheral wells with antigens as indicated below. Abbreviations in parentheses below are as follows:

(Fr) = Freshly prepared extracts, suspended in indicated material.
(W-FD) = Extracts prepared in water, freeze-dried, and suspended in the indicated material just prior to use.
(SDS-FD) = Extracts prepared in SDS, freeze-dried, and suspended in water just prior to use.
(W-FD, 5×) or (SDS-FD, 5×) = Sample freeze-dried a total of five times.

A-PVX antiserum

Medium: SDS-azide agar
Antigens: all from tobacco, suspended in SDS

1 - PVX	(Fr)
2 - PVX	(W-FD)
3 - Healthy	(W-FD)
4 - Healthy	(SDS-FD)
5 - PVX	(W-FD)
6 - PVX	(SDS-FD)

B-TEV antiserum

Medium: SDS-azide agar
Antigens: all from tobacco, suspended in SDS

1 - TEV, type strain	(Fr)
2 - TEV, Oxnard strain	(SDS-FD)
3 - TEV, type strain	(SDS-FD)
4 - TEV, severe strain	(SDS-FD)
5 - Healthy	(Fr)
6 - TEV, severe strain	(SDS-FD)

C-PVY antiserum

Medium: SDS-azide agar
Antigens: all from tobacco, suspended in SDS

1 - PVY	(SDS-FD)
2 - PVY	(SDS-FD, 5×)
3 - PVY	(Fr)
4 - PVY	(W-FD, 5×)
5 - PVY	(W-FD)
6 - Healthy	(Fr)

D-SBMV antiserum

Medium: Tris-azide-NaCl agar
Antigens: from bean or cowpea, suspended in water

1 - SBMV, bean	(W-FD)
2 - SBMV-C, cowpea	(Fr)
3 - SBMV, bean	(Fr)
4 - SBMV, bean	(W-FD)
5 - SBMV-C, cowpea	(W-FD)
6 - Healthy bean	(Fr)

E-TEV antiserum

Medium: SDS-azide agar
Antigens: all from tobacco, suspended in SDS

1 - TEV	(Fr)
2 - PVY	(Fr)
3 - TEV	(SDS-FD)
4 - PVY	(SDS-FD)
5 - Healthy	(Fr)
6 - PVY	(SDS-FD)

F-PVY antiserum

Medium: Tris-azide-NaCl agar
Antigens: all from tobacco, suspended in pyrrolidine

1 - PVY	(Fr)
2 - TEV	(Fr)
3 - PVY	(W-FD)
4 - TEV	(W-FD)
5 - Healthy	(Fr)
6 - PVY	(W-FD)

TABLE 1. List of viruses whose antigenic activities were preserved in freeze-dried plant extracts

Virus	Host	Antigen preparation and minimum storage period prior to testing[a]
Bidens mottle	*Lactuca sativa*	SDS (1 year)
	Nicotiana hybrid[b]	SDS (1 year)
Brome mosaic	*Hordeum vulgare*	W (2 months)
Clover yellow mosaic	*Pisum sativum*	SDS (1 year); W (1 year)
Lettuce mosaic	*L. sativa*	SDS (1 year)
	P. sativum	SDS (1 year)
Papaya mosaic	*Papaya carica*	W (1 month)
Pepper mottle	*Capsicum annuum*	W (4 months)
	N. hybrid	W (1 month)
	N. tabacum	SDS (9 months)
Potato Y	*C. annuum*	SDS (4 months); W (3 months)
	N. tabacum	SDS (1 year); W (1 month)
Potato X	*N. tabacum*	SDS (9 months); W (1 year)
Southern bean mosaic	*Phaseolus vulgaris*	W (1 year)
Southern bean mosaic, cowpea strain	*Vigna unguiculata*	W (1 year)
Tobacco etch	*C. annuum*	W (4 months)
	Datura stramonium	SDS (1 year); W (6 months)
	N. tabacum	SDS (1 year); W (6 months)
Tobacco mosaic	*N. tabacum*	W (5 months)
Turnip mosaic	*N.* hybrid	SDS (1 year)

[a]SDS and W denote extracts prepared in sodium dodecyl sulfate (SDS) and water, respectively, prior to freeze-drying. The figures in parentheses indicate the minimum period that each antigen preparation was stored prior to conducting the tests. The southern bean mosaic and brome mosaic viruses were reconstituted with water and tested in Tris-buffered agar medium. For all other antigens, tests were conducted in the SDS-agar medium; extracts prepared in SDS were reconstituted with water, and extracts prepared originally in water were reconstituted with SDS.

[b]*Nicotiana glutinosa* L. × *N. clevelandii* Gray (2).

(14), or 2.5% ethanolamine (10).

Immunodiffusion tests with SDS-denatured antigens were conducted in agar gels containing 0.5% SDS, 1% sodium azide, and 0.8% Noble agar (4, 11). For tests with SBMV and brome mosaic virus and for pyrrolidine- or ethanolamine-degraded antigens of TEV and PVY, the medium consisted of 0.7% Ionagar No. 2, 0.85% sodium chloride, 0.03% sodium azide, and 0.05 M Tris buffer, pH 7.2. Following addition of reactants, plates were incubated at 25 C for 24-48 hours prior to photography.

RESULTS.—Active virus antigens were detected in reconstituted, freeze-dried plant extracts from each virus-host combination tested (Table 1). The intensities of precipitin lines with freshly prepared extracts (FrV) were usually slightly greater than with virus antigens in freeze-dried extracts (FDV). Reactions of serological identity (no spur formation), however, were obtained when FDV were compared directly to FrV. This type of experiment was done with PVX (Fig. 1-A), TEV (Fig. 1-B), PVY (Fig. 1-C), SBMV (Fig. 1-D), papaya mosaic virus and PeMV.

To determine if repeated freeze-drying influenced reactivity of PVY antigens, extracts from PVY-infected tobacco were freeze-dried, reconstituted, and this process was repeated four times. Samples freeze-dried five times gave reactions of identity to samples freeze-dried only once (Fig. 1-C). Reactions with antigens extracted in water, freeze-dried, and suspended in SDS were sometimes slightly weaker than the reactions obtained with antigens directly extracted in SDS and freeze-dried. TEV and PeMV gave results similar to those obtained with PVY.

Various other tests also indicated that the serological specificities of FDV were the same as those of FrV. Two TEV strains in FDV gave reactions of identity with FrV of the type TEV strain (Fig. 1-B). PVY is known to be serologically related to, but distinct from, TEV (1, 10, 14). Freeze-dried PVY antigens and FrV-PVY antigens cross-reacted with TEV antiserum, and the precipitin lines were spurred over either by FrV- or FDV-TEV (Fig. 1-E). Similarly, FDV-PVY or FrV-PVY resuspended in 2.5% ethanolamine or 2.5% pyrrolidine also cross-reacted with TEV antiserum, but the precipitin lines were spurred over

by the reactions either with FDV-TEV or FrV-TEV. The PVY antiserum used in this study gave reactions of identity with pyrrolidine-degraded FDV-PVY and FrV-PVY (Fig. 1-F) but it did not cross-react either with freshly prepared or freeze-dried TEV, regardless of the denaturant used. The two strains of SBMV, which had been shown to differ antigenically (6), gave expected reactions with either FDV or FrV (Fig. 1-D).

The host species used for preparation of FDV did not appear to alter serological specificity, as FDV of lettuce mosaic virus in pea (*Pisum sativum* L.) and lettuce (*Lactuca sativa* L.) gave reactions of identity. Certain hosts (*Datura, Capsicum* spp.) sometimes gave nonspecific reactions in the SDS immunodiffusion system with both fresh and freeze-dried extracts from healthy or infected tissues. These nonspecific reactions, however, could readily be identified by using proper controls.

DISCUSSION.—The successful use of freeze-dried plant extracts for immunodiffusion tests with antigens of thirteen plant viruses was demonstrated herein. We have used the freeze-dried antigens as controls in serodiagnostic tests, for serological relationship studies (12), and for teaching demonstrations. The reconstituted freeze-dried antigens are convenient to use, are time-saving, conserve greenhouse space, facilitate antigen exchange between investigators, and often can obviate the need for continuous maintenance of viruses in living plant tissues. Furthermore, large quantities of samples possessing good serological activity can be freeze-dried for future use.

Occasionally, field samples (e.g., some samples of lettuce mosaic virus in lettuce or endive) were encountered which gave weak reactions in fresh extracts, but gave negative reactions upon freeze-drying. Likewise, the intensity of precipitin lines from greenhouse-grown plants also was often less with FDV than with FrV. The reduction in intensity was noticed upon testing of samples freeze-dried the previous day, and it was not significantly altered further by storage or by repeated (five times) freeze-drying of PVY, TEV, or PeMV antigens. The reasons for the reduction in precipitin line intensity are not understood, but because it occurred in several instances, it may be anticipated that some antigens may fail to withstand the freeze-drying process. Changes in the antigenic specificity of virus antigens also may accompany the freeze-drying process, although such changes have not been detected in the limited tests conducted so far.

In conclusion, the use of freeze-dried extracts from infected plants appears to have considerable potential as an economical means of maintaining a diverse collection of plant virus antigens for use in immunodiffusion tests.

LITERATURE CITED

1. BATCHELOR, D. L. 1974. Immunogenicity of sodium dodecyl sulfate-denatured plant viruses and plant viral inclusions. Ph.D. Dissertation, University of Florida, Gainesville. 82 p.
2. CHRISTIE, S. R. 1969. Nicotiana hybrid developed as a host for plant viruses. Plant Dis. Rep. 53:939-941.
3. GARNSEY, S. M., and D. E. PURCIFULL. 1969. Serological detection of a citrus virus in leaf extracts from field trees. Proc. Fla. State Hortic. Soc. 82:56-60.
4. GOODING, G. V., JR., and W. W. BING. 1970. Serological identification of potato virus Y and tobacco etch virus using immunodiffusion plates containing sodium dodecyl sulfate. Phytopathology 60:1293.
5. GOODING, G. V., JR., and J. P. TSAKIRIDIS. 1971. Sodium azide as a protectant of serological activity and infectivity of plant viruses. Phytopathology 61:943-944.
6. GROGAN, R. G., and K. A. KIMBLE. 1964. The relationship of severe bean mosaic virus from Mexico to southern bean mosaic virus and its related strain in cowpea. Phytopathology 54:75-78.
7. HAMILTON, R. I. 1965. Lyophilized antiserum in paper disks for sero-diagnosis of barley stripe mosaic. Virology 26:153-154.
8. HOLLINGS, M., and O. M. STONE. 1970. The long-term survival of some plant viruses preserved by lyophilization. Ann. Appl. Biol. 65:411-418.
9. MC KINNEY, H. H., and G. SILBER. 1968. Methods of preservation and storage of plant viruses. Pages 491-501 *in* K. Maramorosch and H. Koprowski, eds., Methods in virology, Vol. IV. Academic Press, New York. 764 p.
10. PURCIFULL, D. E., and G. V. GOODING, JR. 1970. Immunodiffusion tests for potato Y and tobacco etch viruses. Phytopathology 60:1036-1039.
11. PURCIFULL, D. E., E. HIEBERT, and J. G. MC DONALD. 1973. Immunochemical specificity of cytoplasmic inclusions induced by viruses in the potato Y group. Virology 55:275-279.
12. PURCIFULL, D. E., T. A. ZITTER, and E. HIEBERT. 1975. Morphology, host range, and serological relationships of pepper mottle virus. Phytopathology 65: 559-562.
13. SCOTT, H. A. 1961. Serological detection of barley stripe mosaic virus in single seeds and dehydrated leaf tissues. Phytopathology 51:200-201.
14. SHEPARD, J. F., G. A. SECOR, and D. E. PURCIFULL. 1974. Immunochemical cross-reactivity between the dissociated capsid proteins of PVY group plant viruses. Virology 58:464-475.