

Stability of Potato Spindle Tuber Viroid in Freeze-Dried Leaf Powder

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ABSTRACT

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A severe strain of the potato spindle tuber viroid (PSTV) survived up to 6 years in freeze-dried tomato leaves stored at room temperature. In tests to date the mild strains were infectious up to 3 years in tomato and *Scopolia sinensis* tissues. There was no apparent loss of infectivity during storage for 6 years. Freshly freeze-dried material had infectivity similar to that of the freeze-dried material stored

for 6 years. The nucleic acid was extractable from freeze-dried tissues. Analysis of the low-molecular-weight ribonucleic acid, by gel electrophoresis on 20% gels, yielded a well-separated PSTV band in both freeze-dried and nonfreeze-dried tissues. The use of freeze-dried samples facilitates the handling of large amounts of infected material for purification purposes.

Potato spindle tuber viroid (PSTV) is a low-molecular-weight ribonucleic acid (1, 12) that is devoid of protein coat (2, 11). In spite of its small genome size it is capable of infecting many plant species, causing discernible symptoms in some plants and remaining symptomless in others (10). On the basis of its reactions in tomato (*Lycopersicon esculentum* Mill.) plants, two strains have been recognized (4). Both "mild" and "severe" strains of PSTV are encountered in the field (13) and both affect yield differently in potato cultivars (14). Potato spindle tuber viroid is highly infectious, and maintenance of cultures in the greenhouse always raises the possibility of contamination, particularly with the severe strain. Since it was already known that freeze-dried nucleic acids retain infectivity for several months (11), an attempt was made to test the effect on PSTV of freeze-drying infected tissues for extended periods.

The general success of freeze-drying for preserving plant virus infectivity has been well documented (6, 7), but in most cases the freeze-dried materials were stored in sealed containers at low temperatures. Since PSTV has high resistance to heat (11), we wished to know: (i) whether PSTV would survive in freeze-dried leaves in unsealed containers at room temperature; and (ii) whether freeze-dried tissues could be used to concentrate material for purification of RNA on a large scale. The results described in this paper support both possibilities.

MATERIALS AND METHODS

Potato spindle tuber viroid strains used in previous studies (13, 14) [including the mild strains (i) and (ii) supplied by K. H. Fernow and the severe strain (iv) from the Schultz collection] were propagated in tomato

cultivar Sheyenne (11) or in *Scopolia sinensis* Hemsl. plants (10). The infected tissues were harvested 3-4 weeks after inoculation, when both mild and severe strains had become systemic in tomato and *S. sinensis* plants.

For freeze-drying, the leaves from infected and healthy plants were placed in 500-ml VirTis vacuum bottles and frozen at -22 C for 48 hours. Subsequently, the leaves were freeze-dried for 48 hours using the VirTis apparatus (Research Equipment, Gardiner, N. Y.) at a pressure of 0.05 to 0.1 mm of Hg. A Wiley mill (Arthur H. Thomas Laboratory Apparatus, Philadelphia, Pennsylvania), fitted with a 0.5-mm (40-mesh) screen was used to grind the freeze-dried leaves to a fine powder which was stored at room temperature (24-28 C).

Infectivity was tested on three host plants: *S. sinensis*, tomato cultivar Sheyenne, and potato cultivar Netted Gem. Only *S. sinensis* plants were used for quantitative tests. For the infectivity test, 0.5 to 1.0 g of leaf powder was triturated in 2.5 to 5.0 ml of 0.1 M K_2HPO_4 buffer, pH 7.0. The homogenate was mechanically inoculated to test plant leaves that had been dusted with 0.22- μ m (600-mesh) Carborundum powder.

The nucleic acid was extracted as follows: to 10 g of freeze-dried leaf powder were added 150 ml of glycine buffer (3) [0.1 M glycine, 0.1 M NaCl, 0.01M ethylenediaminetetraacetic acid (EDTA), 1% sodium lauryl sulfate (pH 9.5)], 5 ml of bentonite solution (12 mg/ml), and 300 ml of water-saturated phenol. The mixture was stirred at room temperature for 30 minutes. After centrifugation for 10 minutes at 10,000 g, the aqueous layer was removed, extracted with ether (four times), and the nucleic acid was precipitated with 3% cetyltrimethyl ammonium bromide (CTAB) as described previously (12). The CTAB was removed by washing two times with 70% ethanol containing 1 M potassium acetate (16). The low-molecular-weight nucleic acid was obtained by fractionation with 2 M LiCl (12), solution in 0.3 M sodium acetate buffer pH 7.0, and centrifugation (2,000 g,

10 minutes) to remove undissolved material. The nucleic acids were precipitated from the supernatant solution with 2.5 volumes of ethanol and dissolved in electrophoresis buffer, which was the same as described previously (15) except that sodium lauryl sulfate was omitted. The electrophoretic run was 16-20 hours at 8 mA/gel. After electrophoresis, gels were stained with 0.1% toluidine blue O for 6-16 hours, destained in tap water, and the desired bands were cut out for infectivity tests and extraction.

RESULTS

Survival of infectivity in freeze-dried tissues.—Eleven samples (50 g each) of tomato leaves infected with the severe strain of PSTV, freeze-dried in 1969, and stored at room temperature for various periods, remained infectious when tested after 1, 3, and 6 years of storage. In 1972, mild strains (i) and (ii) (13, 14) were included from infected leaves of tomato and *S. sinensis*. Table 1 shows the infections obtained from four samples of severe PSTV and two samples each of the mild PSTV strains, on different host plants. To date, the severe PSTV samples have survived at least 6 years and those of the mild strain have survived for 3 years. All the potato plants infected with severe PSTV developed severe stunting, necrosis of stems, and reduced leaves; whereas the growth habit of those infected with the mild strains was upright and slightly dwarfed. Both strains produced spindly tubers. PSTV was recovered from potato leaves and tubers on *S. sinensis* plants. Symptoms on tomato were typical of the strains (4, 13). *Scopolia sinensis* plants inoculated with crude tissue extract developed typical local lesions, although their number varied among samples (Table 1).

Comparison of infectivity in freshly freeze-dried and 6-year-old freeze-dried tissues.—The nucleic acid isolated from 10 g of tissue either freshly freeze-dried or freeze-dried 6 years previously, were used for infectivity comparison. The yield of low-molecular-weight fraction (2 M LiCl-soluble) from both preparations was about 100 A₂₆₀ units. Both preparations were diluted equally with 0.1 M K₂HPO₄ buffer, pH 7.2, before inoculation to *S. sinensis* leaves. As shown in Table 2, there was no loss of

infectivity of the viroid in 6-year-old material compared to that in the freshly freeze-dried sample.

Use of freeze-dried material for large-scale purification.—Several buffer and homogenizing procedures were tried with freeze-dried tissue powder. Use of sodium deoxycholate or short stirring periods failed to extract any RNA. The use of glycine buffer as described by Diener and Smith (3) extracted RNA in greater amounts than did other buffers that were tried, although most preparations were viscous and failed to migrate sharply on electrophoretic gels. However, when such RNA preparations were dissolved in 5-10 ml of 0.3 M sodium acetate buffer, pH 7.0, centrifuged at low speed to remove undissolved material, and precipitated with 2.5 volumes of ethanol to obtain RNA the viscosity was removed without affecting PSTV concentration. The preparations from potato and tomato tissues were pigmented, whereas those from *S. sinensis* were not.

The electropherogram of purified low-molecular-weight RNA (LMW-RNA) from *S. sinensis* is shown in Fig. 1. The RNA's were extracted from fresh (nonfreeze-

TABLE 2. Comparison of the infectivity of nucleic acids from tomato leaf tissue infected with the potato spindle tuber viroid either freshly freeze-dried or freeze-dried and stored at room temperature for 6 years

Nucleic acid concentration (A ₂₆₀)	Average number of local lesions ^a	
	Fresh ^b	Stored ^c
10.0	174	156
1.0	69	72
0.1	39	38
0.01	9	8

^aAverage number of local lesions from eight half-leaves of *Scopolia sinensis* plants from one test. The test inoculum was on four left and four right half-leaves of eight plants. Both RNA samples were compared on each plant. A ~ 50 µliter sample was used to inoculate each half-leaf.

^bTomato tissues infected with potato spindle tuber viroid were freeze-dried 2-3 days prior to nucleic acid extraction.

^cTomato tissues freeze-dried and stored for 6 years.

TABLE 1. Residual infectivity of freeze-dried leaf tissues from plants infected with the potato spindle tuber viroid after storage at room temperature for 3-6 years.

Viroid strain and source plant	Storage periods (years)	Infectivity in different plants		
		Potato ^a	Tomato	<i>Scopolia sinensis</i>
Severe PSTV #8 Tomato	5	4/4 ^b	3/3	83 ^c
Severe PSTV #9 Tomato	5	4/4	3/3	90
Severe PSTV #10 Tomato	5	4/4	3/3	56
Severe PSTV #11 Tomato	6	4/4	3/3	83
Mild (i) PSTV Tomato	3	4/4	7/7	15
Mild (i) PSTV <i>Scopolia</i>	3	4/4	3/7	8
Mild (ii) PSTV Tomato	3	4/4	7/8	49
Mild (ii) PSTV <i>Scopolia</i>	3	4/4	7/8	40
Severe PSTV (Tomato, freshly prepared)	0	4/4	3/3	29

^aPotato plants of cultivar Netted Gem were grown from indexed stem cuttings free of visible and latent viruses, and of fungal and bacterial pathogens. They developed visible symptoms within 4-5 weeks after inoculation with mild and severe PSTV.

^bThe numerator represents the plants infected, and the denominator represents the number of plants inoculated.

^cAverage number of local lesions from four half-leaves of *S. sinensis* plants.

dried) healthy, fresh PSTV-infected, and freeze-dried PSTV-infected tissues. One hundred grams of fresh leaf tissue or equal fresh leaf tissue after freeze-drying (about 10 g) were used for RNA extraction. The yield of LMW-RNA ranged between 110-120 units of A_{260} from these three preparations; thus, freeze-dried preparations contained as much RNA as the fresh ones. In order to determine if the amount of PSTV was altered, the RNA preparations were electrophoresed on 10%, 15%, and 20% gels. Ten A_{260} units applied on each gel (20%) are depicted in Fig. 1. The PSTV band was well separated in both nonfreeze-dried (Fig. 1-A) and freeze-dried (Fig. 1-C) PSTV-infected tissue preparations and was absent in the preparation from healthy tissue (Fig. 1-B). Thus, it appears that there was no significant loss of PSTV during freeze-drying of the tissues, because both preparations had similar PSTV bands. Similar results were obtained on 10% and 15% gels (Singh and Finnie, *unpublished*).

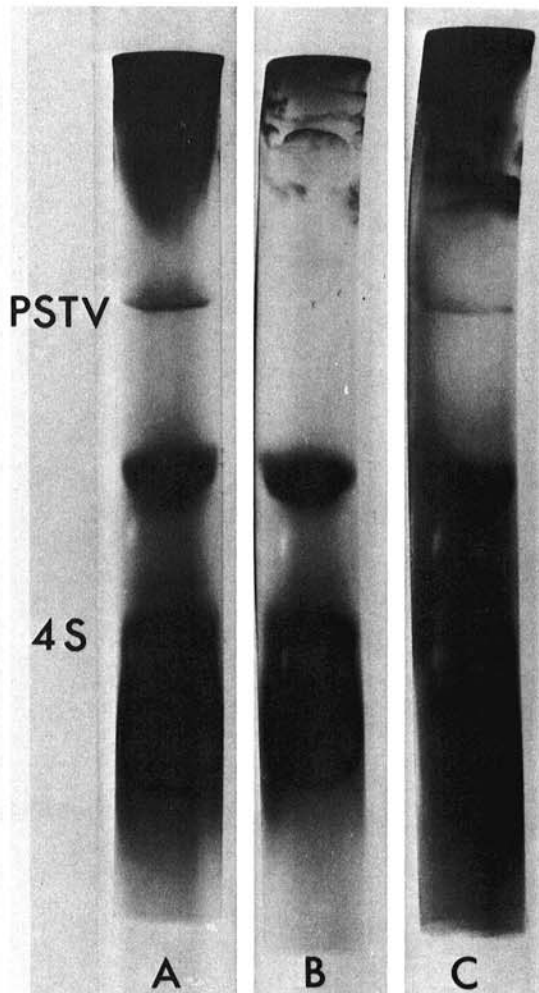


Fig. 1-(A to C). Electropherogram of preparations from *Scopolia sinensis*: A) nonfreeze-dried preparation from the potato spindle tuber viroid-infected plants; B) nonfreeze-dried preparation from healthy plants; and C) freeze-dried preparation from the potato spindle tuber viroid-infected plants.

The identity of the PSTV band was confirmed by excising the stained band, grinding the gels in 0.1 M phosphate buffer, pH 7.0, and mechanically inoculating it into *S. sinensis* leaves. From the typical run presented (Fig. 1), bands denoted "PSTV" produced an average of 156 and 143 lesions and the band marked "4S" produced an average of 20 and 11 lesions from nonfreeze-dried and freeze-dried preparations, respectively. No lesions resulted from any other bands of infected preparations and none from preparations from healthy plants (Fig. 1-B).

DISCUSSION

The remarkable stability of PSTV-RNA at high temperature (11) and its survival in dried true seeds of potato (5) and tomato (9) are well known; therefore, it was not unexpected to find that PSTV survived 6 years in freeze-dried tissues stored at room temperature in unsealed containers. The fact that both mild and severe strains can survive in freeze-dried tissues, makes it easier to store cultures free of contamination. Extended storage at room temperature had no effect on infectivity: both freshly freeze-dried and stored material had similar infectivity.

The viroid RNA comprises a very small part (0.001 to 0.01%) of the total nucleic acid extracted from the host plant (8). Thus, a large amount of infected material is needed to provide sufficient quantity of viroid RNA for biochemical studies. Since freeze-drying reduces the weight of fresh infected plant material to approximately one-tenth, extraction can be scaled up to five times without straining centrifugation and other purification facilities.

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