Effect of Storage Temperatures on Potato Virus Infectivity Levels and Serological Detection by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Leaves infected with potato leafroll virus or potato virus A, S, X, or Y stored at 25, 4, −20, or −70 C produced variable infectivity and serological readings. Freezing at −20 C lowered the A_{405} values in ELISA for all potato viruses tested except PVX. Infectivity levels of PVA and PVY were also lowered at −20 C. Refrigeration of leaves at 4 C or freezing at −70 C preserved the infectivity and serological activity up to 12 days. The lower A_{405} values at −20 C were not due to dilution effect, were not affected by host species, and were similar for stored leaves and expressed sap. Purification of PVY from leaves stored at 4, −20, and −70 C produced the lowest yield of virus at −20 C and the highest at −70 C. Pretreatment of leaves in solutions of 5% yeast extract, 5% bactotryptone, 5% bactopeptone, 10% sucrose, or 10% glycine followed by storage at −20 C partially retained both infectivity and serological activity.

One effective method of controlling virus diseases in potatoes (*Solanum tuberosum* L.) is the use of virus-free propagating material. For maintaining freedom from viruses, accurate and rapid identification of viral pathogens is essential to seed certification programs. Plant virus detection by enzyme-linked immunosorbent assay (ELISA) (3) has been applied to potato viruses (5,8,11,15) with varying success. Before an effective seed certification system based on ELISA can be established, the conditions for handling and storing large numbers of leaf samples that will be indexed during the growing season must be studied.

The effect of freezing temperature (−14 to −20 C) on the storage of virus-infected leaves has been variable. Freezing temperature lowered the serological values for potato virus Y (PVY) (16) and plum pox virus (1) but had no effect on ELISA values obtained with apple chlorotic leaf spot virus (4) or PVY (6). With peach rosette mosaic virus, storing grape leaf tissue in the refrigerator for 2 or 3 days lowered the sensitivity of ELISA detection; freezing expressed sap in glass vials at −20 C gave more satisfactory results, however (10). Thus, in the absence of precise information on the effect of freezing temperature on potato viruses, we considered it essential to study the effect of storage temperatures on virus infectivity levels and serological detection by ELISA. A preliminary report appeared elsewhere (13).

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MATERIALS AND METHODS

Viruses. PVY and potato leafroll virus (PLRV) were isolated from commercial fields of the potato cultivar Russet Burbank. PLRV was transferred to and maintained by *Myzus persicae* in potato and *Physalis floridana* plants. Infected *P. floridana* plants were maintained in a 27 C growth chamber with light intensity of 6 klux and a photoperiod of 14 hr. PVY was maintained in potato cultivars Saco and Russet Burbank as well as in tobacco (*Nicotiana tabacum* 'Samsun'). Potato virus A (PVA) and potato virus X (PVX) were maintained in Russet Burbank and potato virus S (PVS), in USDA seedling 41956. In addition to greenhouse cultures, leaf samples were obtained from commercial fields.

Immunological procedures. Antisera

against PVA and PVY were produced locally using the procedure described for PVA (15). Antisera for PVX and PVS were obtained from J. G. McDonald, Research Station, Charlottetown, Prince Edward Island, Canada. Antiserum against PLRV was obtained from R. Stace-Smith, Research Station, Vancouver, BC. Anti-γ-globulin was isolated by double precipitation of antisera with saturated ammonium sulfate (v/v) and desalting the γ-globulin on Sephadex G-25M columns (Pharmacia Fine Chemicals, Dorval, Quebec) or dialysis and filtration through DEAE-cellulose DE-22 columns (3). The γ-globulin in PBS (0.02 M phosphate-buffered saline + 0.2% sodium azide) was conjugated with alkaline phosphatase (Type VII, Sigma Chemicals Co., St. Louis, MO 63178). For conjugate preparation, the enzyme was centrifuged at 8,000 *g* for 10 min and the precipitate dissolved in 2 ml of γ-globulin (enzyme to γ-globulin ratio 2:1, w/w). The mixture was dialyzed at 4 C for 24 hr, with three changes of buffer, followed by dialysis for 8 hr against PBS buffer containing 0.2% glutaraldehyde at 25 C. Excess glutaraldehyde was removed by dialysis at 4 C for 24 hr, with three changes of PBS buffer.

Table 1. Effect of storage at various temperatures on infectivity of potato leaves infected with potato virus A or Y

Virus	Local lesions[a]			
	25 C	4 C	−20 C	−70 C
PVA	58.6	33.8	0.4	42.8
PVY	248.7	128.0	6.5	92.8

[a] Average number of local lesions from 20 leaves per experiment.

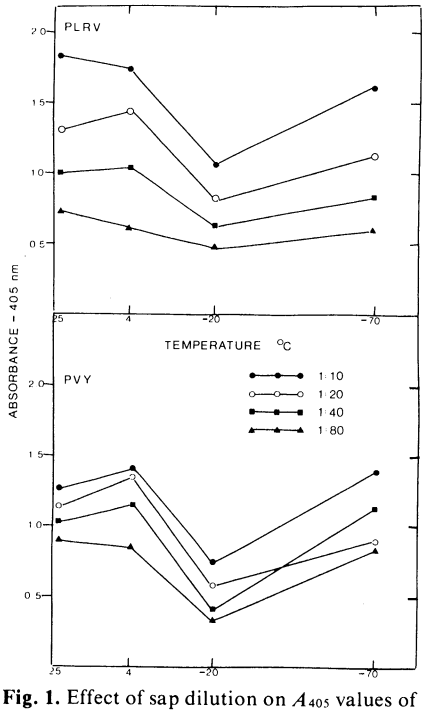

Fig. 1. Effect of sap dilution on A_{405} values of potato leaves infected with potato virus Y (PVY) or potato leafroll virus (PLRV), stored at various temperatures for 4 days, then ground and diluted in grinding buffer.

ELISA procedures, using flat-bottomed polystyrene microtitration plates (Linbro, Flow Laboratories, Inc., Mississauga, Ontario) were performed as previously described (3,15). Plates were coated with γ-globulin (1 μg/ml), test samples were incubated at 4 C overnight, conjugate was applied at 1/400 dilution (2.5 μg/ml), and absorbance values were measured at 405 nm (A_{405}) 1 hr after the alkaline phosphatase substrate was added, with a Titertek Multiskan (Flow Laboratories). Each sample was placed in two adjacent wells in each test. Each experiment was repeated at least five times with positive and negative controls included.

Preparation of leaf samples. Leaf samples (1 gm fresh weight) were collected and stored at 4 C, −20 C, or −70 C. For comparison, fresh samples (25 C) of diseased leaves were used. Negative healthy leaf controls were stored at the same temperatures. Leaf samples were ground in grinding buffer (PBS + 0.05% Tween 20 + 2% polyvinylpyrrolidone) in a ratio of 20:1 (v/w), using a Polytron homogenizer with PT-10 generator (Brinkmann Instruments [Canada] Ltd., Rexdale, Ontario). Infectivity was tested for PVA and PVY on local lesion hosts *P. angulata* (14) and *S. demissum* PI 230579 (17).

RESULTS

Preserving virus infectivity or serological identity of plant viruses by freezing at −20 C has long been practiced (2,9) and is still in use (6,7). To determine whether the same ELISA values are obtained from freshly harvested and frozen leaf samples, PLRV- or PVY-infected samples were divided, stored at 25 or −20 C for 1–3 days, and tested by ELISA. The A_{405} for PLRV- and PVY-infected leaves (1/20 sap dilution) was 1.720 and 1.512, respectively. The corresponding A_{405} for healthy samples was 0.232. The infected samples frozen at −20 C, however, gave A_{405} values of 0.612 (PLRV) and 0.652 (PVY). When lower A_{405} values were obtained repeatedly from frozen samples, various possibilities were examined to explain the difference.

Since manual defrost (MD) and frost-free (F-F) refrigerators are used in various laboratories, eight refrigerators of different brands were tested. Four were frost-free and four were manual defrost. Leaves infected with PVY were stored at 4 C (± 2) and −20 C (± 3). After 4 days of storage, samples were tested by ELISA. There was no significant difference between F-F and MD refrigerators. The A_{405} values, however, were higher for leaves stored at 4 C (1.492 F-F, 1.526 MD) and lower at −20 C (0.473 F-F, 0.627 MD).

Since PVY-infected leaves stored at −20 C produced lower A_{405} values, it was of interest to determine if a similar change occurred in virus infectivity. Potato leaves infected with PVA or PVY were taken, divided, and stored at various temperatures. After 4 days of storage at 4, −20, and −70 C, the samples were ground in glycine-phosphate buffer (14,15) and tested for infectivity by inoculation to local lesion hosts. The average number of lesions was significantly reduced at −20 C (Table 1). The infectivity of PVA and PVY was correlated with A_{405} values observed after storage at various temperatures.

To determine whether the lower A_{405} values of infected leaves were due to concentration changes during the freezing process, leaf samples stored for 4 days at 4, −20, or −70 C and freshly harvested (25 C) samples were compared. Expressed sap was diluted with grinding buffer to 1:10, 1:20, 1:40, and 1:80. The A_{405} values were significantly lower at −20 C of each dilution (Fig. 1). The lower values were not due entirely to the freezing process because values were higher at −70 C. The dilution of sap caused a general lowering of A_{405} values for each sample, irrespective of storage temperature. PLRV- and PVY-infected leaves behaved similarly. No change of A_{405} values was observed with healthy leaves stored at various temperatures.

Since differences in ELISA values of frozen leaves or expressed sap have been

Table 2. Effect of storage duration on absorbance values of infected leaves and expressed sap at various temperatures

Virus	Samples	Temp. (C)	Storage time (days)				
			1	3	6	9	12
PLRV	Leaves	4	1.191[a]	1.154	1.050	1.257	1.380
		−20	0.643	0.541	0.495	0.572	0.519
		−70	0.933	0.916	0.784	0.749	0.776
PLRV	Sap	4	1.357	1.281	1.139	1.836	1.578
		−20	0.634	0.797	0.582	0.814	1.175
		−70	0.804	0.883	0.841	0.883	0.828
PVY	Leaves	4	0.956	1.247	1.340	1.146	1.225
		−20	0.438	0.539	0.550	0.637	0.342
		−70	0.851	1.033	1.050	1.039	1.009
PVY	Sap	4	1.373	1.513	1.248	1.261	1.441
		−20	0.407	0.311	0.250	0.357	0.284
		−70	1.117	1.116	0.750	0.959	1.025
	Healthy leaves	4	0.107	0.122	0.126	0.222	0.207

[a] Mean A_{405} values of five experiments.

noted for other viruses (10), this was examined using PLRV- and PVY-infected leaves and expressed sap. Samples were tested after 1, 3, 6, 9, and 12 days of storage at 4, −20, or −70 C. Leaves or expressed sap stored at 4 C or −70 C for up to 12 days had significantly higher A_{405} values than those stored at −20 C (Table 2). There was no difference in A_{405} values obtained from frozen leaves and those obtained from expressed sap at any temperature. No change in A_{405} values was observed with healthy leaves stored at various temperatures.

To determine whether other potato viruses were affected by storage at −20 C, potato viruses A, S, and X were also tested after 4 days of storage by the same methods. Except for PVX, all others (A, S, Y, and leafroll) had significantly lower A_{405} values at −20 C than at 25, 4, or −70 C (Fig. 2).

It was of interest to know whether storage temperature differences could be observed between different plant species infected with the same virus. For this purpose, PVY-infected plants were used. Two hosts with high virus concentration (*P. floridana* and the tobacco cultivar Samsun) and three hosts with lower virus concentration (the potato cultivars Saco and Russet Burbank and the tomato cultivar Sheyenne) were selected. The leaves of all plant species infected with PVY produced significantly lower A_{405} values at −20 C than at other temperatures (Fig. 3). The trend to lower A_{405} at −20 C occurred irrespective of the initial virus concentration in the host plants.

Freezing causes an insufficient cell wall disruption during grinding of tissues, thus preventing the release of virus (12). To determine whether such was the case with PVY-infected leaves stored at various temperatures, virus was purified from leaves stored at 4, −20, and −70 C. Batches of 150 gm of tobacco leaves stored at each temperature were purified by the previously described procedure (15), and the nucleoprotein absorption spectrum was determined by a spectrophotometer (Beckmann DU-8). The nucleoprotein yield differed greatly (Fig. 4). It was lowest at −20 C and highest at −70 C. In another test, purified virus Y was stored at −20 C and tested weekly for infectivity and for A_{405} values. The virus remained infective up to 33 days, and the A_{405} values did not decrease sharply until 40 days.

Several chemical additives are known to aid preservation of viruses (9), and we tried additional chemicals and methods to prepare samples. Leaves were collected from potato plants infected with PVA, PVY, or PLRV, divided, and processed for storage at −20 C. Leaves either were dipped in a particular solution before storage in a plastic bag or were placed in moist or dry bags to change moisture content. Serological activity and infectivity were partially protected by 5% bactotryptone, 5% bactopeptone, 10% sucrose, 5% yeast extract, or 10% glycine (Table 3). The same treatment did not afford protection equally to all the viruses tested, however. Also, the treatments that enhanced or retained viral infectivity (glycine with PVA, albumin 5% with PVY) were not as successful in preserving serological activity as fresh infected leaves.

DISCUSSION

These experiments show that storage at −20 C of leaves or expressed sap containing potato virus A, S, Y, or leafroll is not satisfactory. Both infectivity and serological property are severely lowered, and this method of storing leaves should be avoided for large-scale testing. Our observation of lower PVY titer by storage at −20 C is in agreement with an earlier report (16) but not with a recent one (6). Why this effect at one temperature exists with the same virus is hard to explain at present.

The best way to store potato leaf samples for several days is under refrigeration at 4 C. In our study, neither PLRV nor PVY showed a significant loss of titer during 12 days of storage at this temperature (Table 2). Virus concentration as measured by A_{405} values in frozen leaves remained high up to 12 days at −70 C, but an ultracold freezer in every laboratory may not be practical.

The suggestion that freezing alone results in incomplete release of virus because of insufficient disruption of cell walls (12) is not fully substantiated by our present work. Virus Y yield was very low in leaves stored at −20 C but very high in

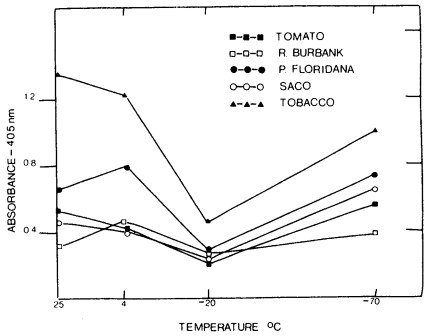

Fig. 3. Effect on A_{405} values of leaves of different host plants infected with potato virus Y and stored at various temperatures.

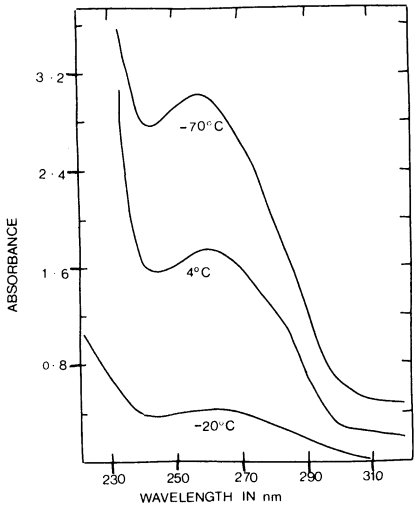

Fig. 4. Yield of nucleoproteins of potato virus Y from tobacco leaves stored at various temperatures.

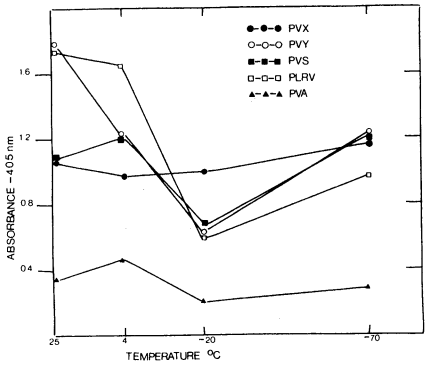

Fig. 2. Effect on A_{405} values of leaves infected with potato virus X (PVX), Y (PVY), S (PVS), leafroll (PLRV), or A (PVA), stored at various temperatures.

Table 3. Effect of various treatments on absorbance values of virus-infected leaves stored at −20 C for 4 days

Treatment	A_{405}			Local lesion[a]	
	PLRV	PVA	PVY	PVA	PVY
Fresh infected leaves	1.971[b]	1.116	1.745	15	40
Bactotryptone 5%	1.685	0.746	1.182	17	5
Sucrose 10%	1.461	0.790	0.872	2	2
Yeast extract 5%	1.443	0.783	1.101	1	0
Glycerin 5%	1.409	0.729	1.176	0	0
Bactopeptone 5%	1.335	0.766	0.884	8	2
Glycine 10%	1.322	0.781	0.948	35	7
Dry paper	1.127	0.627	0.815	2	2
Wet bag	1.005	0.594	0.678	1	2
Dry bag (standard)[c]	0.909	0.638	0.644	0	2
Wet paper	0.907	0.560	0.802	1	11
Albumin 5%	0.889	0.643	0.844	0	15
Healthy leaves, dry bag	0.192	0.153	0.198	...	...

[a] Average number of local lesions from five leaves per experiment.
[b] Mean A_{405} values of five experiments.
[c] Leaves were stored in plastic bags at a particular temperature in each experiment throughout the study.

those stored at −70 C, although both temperatures resulted in frozen leaves. Similarly, samples of expressed sap (tissue disruption before freezing) containing PVY or PLRV stored at −20 C had lower A_{405} values, but not those stored at −70 C (Table 2). This indicates that factors other than virus extractability alone may be responsible for low infectivity or serological activity at −20 C. The difference between −20 and −70 C, on the other hand, may reflect the differential effect of slow vs. fast freezing, which is well known for many physiological processes. Probably the complex medium of cell sap contains inactivating substances that destroy virus infectivity or serological activity at slow freezing temperatures. This contention is supported partly by the evidence that purified virus remained infectious up to 33 days and did not lose A_{405} values for up to 40 days. That some chemical dips were able to protect serological activity and infectivity (Table 3) strengthens this argument.

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