Strategy for Detecting Low Levels of Potato Viruses X and S in Crops and Its Application to the Victorian Certified Seed Potato Scheme

JANE R. MORAN, R. G. GARRETT, and JANET V. FAIRWEATHER, Plant Research Institute, Department of Agriculture, Burnley, Victoria, 3121, Australia

ABSTRACT

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A strategy for detecting low levels of virus infection is described. It was evaluated by studying the incidence of potato virus X (PVX) and potato virus S (PVS) in the Victorian Certified Seed Potato Scheme, using pooled samples and enzyme-linked immunosorbent assay (ELISA). In 57,000 plants examined for both PVS and PVX, incidence for both viruses averaged 0.13%. ELISA was able to detect one infected plant in 1,000.

In order to detect low levels of virus infection in crops, many plants need to be tested. The workload can be decreased by pooling samples, but the consequent diluent effect on infected samples requires a very sensitive testing procedure.

Wright et al (9) used the latex test on pooled samples to test for virus in potato crops; however, enzyme-linked immunosorbent assay (ELISA) is more sensitive than the latex test (3) so the size of pooled samples can be increased.

This paper outlines a strategy for detecting low levels of virus infection, which was evaluated by investigating the incidence of potato virus X (PVX) and potato virus S (PVS), using pooled samples and ELISA, in the Victorian Certified Seed Potato Scheme (4).

MATERIALS AND METHODS

Sampling. In the 1979–1980 season, plantings of foundation seed (four field generations after initial virus testing) and certified seed (six field generations) were sampled by collecting one terminal leaflet from each of 1,000 plants at random throughout each planting. Leaflets were frozen in batches of 200 that were halved at testing so that 10 batches of 100 leaflets were tested per planting. Sampling was similar in 1980–1981, except leaflets were divided into 20 batches of 50 leaflets.

ELISA. All tests were done on polystyrene microtiter plates (2) with antisera produced by R. H. Taylor at the Plant Research Institute, Victoria. Each batch of leaflets was homogenized in two volumes (w/v) of phosphate-buffered saline, pH 7.4, containing 0.5% Tween 20 and 2% polyvinylpyrrolidone (mol wt 44,000) (PBS-Tween-PVP), squeezed through muslin, and 5 ml was frozen until tested. Reactions were assessed visually and spectrophotometrically at 405 nm after reactions were stopped by adding 3 M sodium hydroxide.

Samples were first tested simultaneously for PVX and PVS on the same plate. Those with positive results were then tested separately against each antiserum. When both viruses were tested on the same plate, the plates were coated with a mixture of both virus γ-globulins, each at 0.5 $\mu g\ ml^{-1}$, and mixtures of the enzyme-antibody conjugates were used, each at a dilution of 1/200. When assays for PVX and PVS were done separately, individual plates were coated with γ-globulin at 1 $\mu g\ ml^{-1}$ and the enzyme-antibody conjugate was used at a dilution of 1/200. Test samples were considered positive when absorbance at 405 nm was twice the average for the negative controls.

Estimation of field infection levels. Field infection levels were estimated from the proportions of batches of samples that contained infected leaves. This was done by deriving the proportion of leaves not infected (q) from the proportion of batches not infected (Q) using

$$q = Q^{\frac{1}{N}}, \qquad (1)$$

where N is the number of plants per batch. The proportion of leaves infected (p) was derived using

$$p = 1 - q. \qquad (2)$$

Confidence limits were obtained for Q from the tables of Rolf and Sokal (7). These were translated into confidence limits for p by applying equations 1 and 2 to the appropriate upper and lower confidence limits of Q. This was done for all possible outcomes of the two tests used, ie, 10 batches of 100 leaves and 20 batches of 50 leaves. Estimates of field infection levels and the corresponding 95% upper and lower confidence limits are presented in Tables 1 and 2.

Assumptions made were 1) that the N plants were sampled once and chosen at random, 2) that the sample from an infected plant contained viral antigen, and 3) that the test for virus was completely effective.

RESULTS

ELISA. Positive controls, consisting of a disk of leaf tissue taken with a 15-mm cork borer, infected with PVS and/or PVX, and homogenized in PBS-Tween-PVP with 99 similar disks of healthy leaf tissue, and negative controls, consisting of similar extracts of healthy potato leaves, were included on each ELISA plate. Positive controls gave a strong reaction but negative controls gave

Table 1. Estimates of field infection levels and their 95% confidence limits, based on virus infection in 10 batches of 100 leaflets each

Number of batches contaminated	Estimated field infection (%)	95% Confidence limits	
		Minimum	Maximum
0	0	0	0.363
1	0.105	0.003	0.587
2	0.223	0.026	0.809
3	0.356	0.069	1.050
4	0.509	0.130	1.330
5	0.691	0.207	1.663
6	0.912	0.303	2.082
7	1.197	0.427	2.671
8	1.597	0.585	3.614
9	2.276	0.806	5.815
10	100	1.169	100

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almost no reaction (Table 3), indicating that the test would detect one infected leaf in a batch of 100 leaves. Further tests showed that one PVX-infected leaf in a batch of 1,000 leaves and one PVS-infected leaf in 300 gave visual reactions.

Field infection levels. In the 1979–1980 season, nine foundation and 20 certified seed plantings were tested (Table 4). PVX was not detected in any of the foundation seed plantings tested, whereas PVS was detected in two. Neither virus was found in plantings of Sequoia, Kennebec, Tasman, Sebago (two plantings), and Exton C (two plantings). PVS was present in an estimated 0.105% of one Coliban planting and in 100% of an Exton B planting known to be universally infected.

Neither virus was detected in 10 of the certified seed plantings tested: Pontiac (one planting), Coliban (one planting), Kennebec (one planting), Sequoia (one planting), and Sebago (six plantings). Five plantings had PVS alone, ranging from an estimated 0.1 to 2.28%. Two plantings had dual infections: a Coliban planting had an estimated 0.22% PVX and 0.36% PVS and an Exton B planting known to be universally infected with PVS had an estimated 1.6% PVX.

In the 1980–1981 season, seven foundation and 21 certified seed plantings were tested (Table 5). Of the foundation seed plantings, PVX was present in an estimated 0.21% of one Delaware planting and PVS in an estimated 0.21% of one Coliban planting. Neither virus was found in plantings of Pontiac, Sequoia, Sebago, Kennebec, or Coliban.

Neither virus was detected in 11 of the certified seed plantings tested: Pontiac (one planting), Tasman (one planting), Sequoia (three plantings), Sebago (three plantings), and Kennebec (three plantings). Two plantings had PVS alone: Coliban had an estimated 2.08% and Pontiac had an estimated 0.33% incidence; and six plantings had PVX alone, ranging from an estimated 0.1 to 1.19% incidence. Two plantings had dual infections: a Coliban planting had an estimated 0.57% PVX and 1.19% PVS and an Exton B planting known to be universally infected with PVS had an estimated 0.57% PVX.

DISCUSSION

In order to monitor infection rates in certification schemes, a suitable method is required to test the numerous samples necessary to detect low levels of infection. ELISA meets this requirement. At present, with incidences generally below 1%, testing 10 batches of 100 leaves each has proven effective with the Victorian Certified Seed Potato Scheme. However, if infection levels are reduced to the more desirable 0.1% or less, a considerable increase in total sample size will be necessary.

In a similar potato certification scheme, Wright et al (9) considered it necessary to reexamine batches that contained virus to determine precisely the number of infected leaflets in them. However, our data suggest that retesting is not necessary provided that not all of the batches are infected. Indeed, provided that infection levels are less than about 2%, there is little advantage in distributing the 1,000 leaflets among 20 rather than 10 batches (Tables 1 and 2). The extra labor of testing would probably be better directed at examining further samples to detect infection below the threshold of the current tests.

The strategy of choosing the number of samples for assay warrants discussion. Equation 1 can be used to estimate the probability that a test (as opposed to a batch) composed of N samples would contain at least one infection. Thus, if a test is required to detect 0.1% infection 95% of the times the test is applied, we have: $0.05 = 0.999^N$ or $\log(0.05) = N(\log 0.999)$ and $N = 2{,}994$, which is independent of planting size. Samples can then be distributed among, say, 10 batches so that an estimate of the infection level (as opposed merely to virus detection) can be obtained.

For this type of sampling, an ELISA is needed that can detect low levels of virus in a batch. PVS has previously only been reliably detected in batches of 10 (1) and in batches of 10–20 leaves (6), and PVX has only been reliably detected in batches of 100 leaves (1). Our test for both viruses reliably detected one infected leaf in 1,000 for PVX and one in 300 for PVS. Three isolates of both PVX and PVS were tested and some strains of PVS may have escaped detection; however, ELISA is used routinely to confirm electron microscope diagnosis, which it has never failed to do.

Seven potato varieties were tested from four potato-growing districts in Victoria: Ballarat, 110 km northwest of Melbourne; Kinglake, 50 km northeast of Melbourne; The Otways, 160 km southwest of Melbourne; and Thorpedale, 140 km southeast of Melbourne. We found no correlation between disease incidence and either variety, district, or grower. This may have been due to the low levels of virus detected and a larger survey may dispute this.

Results indicate that infection increased with the number of years the potatoes were grown in the field. For example, plantings of four field generations (foundation seed) had an average of 0.013% PVX and 0.021% PVS, whereas plantings of six field generations (certified seed) had an average of 0.173% PVX and 0.173% PVS. The level of PVS in Victorian seed potatoes should drop as the Exton C line (PVS-free) (8) released in 1981 replaces Exton B.

Tolerance levels for all viruses in the Victorian Certified Seed Potato Scheme are 0.05% for foundation seed and 1% for

Table 2. Estimates of field infection levels, and their 95% confidence limits, based upon virus detection in 20 batches of 50 leaflets each

Number of batches contaminated	Estimated field infection (%)	95% Confidence limits	
		Minimum	Maximum
0	0	0	0.368
1	0.103	0.003	0.569
2	0.211	0.025	0.759
3	0.325	0.065	0.949
4	0.445	0.108	1.141
5	0.574	0.181	1.343
6	0.711	0.253	1.554
7	0.859	0.333	1.777
8	1.016	0.423	2.020
9	1.189	0.523	2.283
10	1.377	0.633	2.570
11	1.584	0.754	2.892
12	1.815	0.890	3.257
13	2.078	1.043	3.675
14	2.379	1.217	4.168
15	2.734	1.411	4.771
16	3.168	1.644	5.552
17	3.723	1.920	6.647
18	4.502	2.271	8.406
19	5.815	2.746	12.45
20	100	3.499	100

Table 3. Enzyme-linked immunosorbent assay absorbance values at 405 nm of potato leaf extracts infected with potato virus X (PVX) and potato virus S (PVS) and controls consisting of healthy potato leaf extracts and phosphate-buffered saline (PBS)

Virus	Mean[a]	Standard deviation
PVX[b]	2.802	0.708
PVS[b]	0.972	0.174
PVX + PVS[b]	3.486	0.870
Healthy potato leaves	0.148	0.072
PBS control	0.114	0.036

[a] Mean of 34 tests.
[b] Each consisting of one potato leaf disk infected with PVX and PVS per 100 healthy potato leaf disks.

Table 4. Results of 1979–1980 field surveys showing estimates of field infection levels and their 95% confidence limits for potato virus X (PVX) and potato virus S (PVS)

	PVX			PVS		
Crop sampled[a]	Estimated infection in 10 batches of 100 leaflets (%)	95% Confidence limits Minimum	Maximum	Estimated infection in 10 batches of 100 leaflets (%)	95% Confidence limits Minimum	Maximum
Foundation seed						
Coliban	0	0	0.363	0.105	0.003	0.587
Exton B	0	0	0.363	100	1.169	100
Certified seed						
Coliban	0	0	0.363	2.276	0.806	5.815
Coliban	0	0	0.363	0.105	0.003	0.587
Coliban	0.223	0.026	0.809	0.356	0.069	1.050
Exton B	1.597	0.585	3.614	100	1.169	100
Katahdin	0	0	0.363	0.223	0.026	0.807
Kennebec	0	0	0.363	0.105	0.003	0.587
Sebago	0	0	0.363	0.105	0.003	0.587
Sebago	0.105	0.003	0.587	0	0	0.363
Sebago	0.691	0.207	1.663	0	0	0.363
Sebago	0.691	0.207	1.663	0	0	0.363

[a] Seven foundation seed crops: Sequoia, Tasman, Kennebec, two Sebago crops, and two Exton C crops; and 10 certified seed crops: Pontiac, Coliban, Kennebec, Sequoia, and six Sebago crops, were free of both PVS and PVX.

Table 5. Results of 1980–1981 field surveys showing estimates of field infection levels and their 95% confidence limits for potato virus X (PVX) and potato virus S (PVS)

	PVX			PVS		
Crop sampled[a]	Estimated infection in 20 batches of 50 leaflets (%)	95% Confidence limits Minimum	Maximum	Estimated infection in 20 batches of 50 leaflets (%)	95% Confidence limits Minimum	Maximum
Foundation seed						
Coliban	0	0	0.368	0.211	0.025	0.760
Delaware	0.211	0.025	0.760	0	0	0.368
Certified seed						
Coliban	0	0	0.368	2.078	1.043	3.675
Coliban	0.103	0.003	0.569	0	0	0.368
Coliban	0.574	0.181	1.343	0	0	0.368
Coliban	0.574	0.181	1.343	1.189	0.523	2.283
Exton B	0.574	0.181	1.343	100	3.499	100
Pontiac	0	0	0.368	0.325	0.065	0.949
Sebago	0.103	0.003	0.569	0	0	0.368
Sebago	0.445	0.108	1.141	0	0	0.368
Sebago	1.189	0.523	2.283	0	0	0.368
Sebago	0.211	0.025	0.760	0	0	0.368

[a] Five foundation seed crops: Pontiac, Coliban, Sequoia, Sebago, and Kennebec; and 11 certified seed crops: Pontiac, Tasman, three Sequoia crops, three Sebago crops, and three Kennebec crops, were free of PVS and PVX.

certified seed and are based only on visual inspections. Of the 16 foundation seed plantings tested, only four had virus infections greater than 0.05%; three had more than 0.05% PVS (including Exton B, known to be 100% infected) and one had more than 0.05% PVX. Of the 41 certified seed plantings tested, only seven plantings had more than 1% virus infection; five had more than 1% PVS (including two Exton B plantings) and two had more than 1% PVX.

In 1979–1980, PVS was more prevalent than PVX, but in 1980–1981, the reverse occurred. In the two seasons, however, only three crops (excluding Exton B) had more than 1.19% PVS and/or PVX. This result differs from that of a recent survey done in Queensland (5), where as high as 60% PVS and 50% PVX were detected in certified seed potatoes imported from Victoria, by examination of sap-dip preparations with the electron microscope. Their results could perhaps be due to the small numbers of tubers tested (only 20 tubers were collected from each of 11 seed lots). However, in view of the much lower levels of virus recorded in our survey, other explanations seem necessary, perhaps involving spread during storage or handling.

Results from a similar survey conducted in Canada in 1967 (9) showed that infection with PVX and PVS occurred at a considerably higher rate than in Victoria. Wright et al (9) estimated that neither virus was detected in only 91% of Elite 2 plants (generation 3), 88% of Elite 3 plants (equivalent to Victorian foundation seed), and 36% of foundation acreage (generation 5).

The strategy described in this paper has shown that levels of PVS and PVX are acceptably low; however, a wide range of serotypes may be expected in nature and reliance on serological testing, particularly in the early stages of the scheme, could fail to detect new or rare serotypes and lead to their inadvertent propagation. Therefore, there are no plans to incorporate such tests into routine inspection procedures. We have not yet encountered such serotype variation in routine diagnosis so we believe our observations are reliable for this survey. In fact, it is likely in tests on field infected plants that late infections would lead to underestimation of infection levels more often than serotype variation.

The same strategy is used for surveys of other industries served by pathogen-tested stock schemes and could also be used to study other problems where large populations need to be examined for low incidences of virus, in particular, surveys for virus in weed reservoirs, in vector populations, and in quantities of seed. In our experience, the major problems encountered with this strategy were collection of the large numbers of leaf samples and preparation of homogenates from pooled samples.

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