Survey of New South Wales Potato Crops for Potato Spindle Tuber Viroid with Use of a ^{32}P-DNA Probe

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ABSTRACT

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The dot-blot membrane hybridization assay, which employs a ^{32}P-labeled complementary deoxyribonucleic acid probe, was tested as a means of detecting potato spindle tuber viroid (PSTV) in bulked leaf samples from potato (*Solanum tuberosum*). Levels as low as one infected leaf disk in 400 were detected. Inoculating tomato (*Lycopersicon esculentum*) plants and assaying these with the probe was less reliable and no more sensitive. The direct assay was used in a statewide survey of certified seed potato crops, and it was concluded that none of the 18,900 plants sampled was infected with PSTV.

In 1982, potato spindle tuber viroid (PSTV) was found in the potato breeder's collection of the N.S.W. Department of Agriculture at Glen Innes (9). Because this was the first record of PSTV in Australia, the breeder's collection was destroyed together with lines derived from Glen Innes that were being performance-tested in New South Wales and other states. However, a number of the lines had been used in trials on commercial properties between 1940 and 1982, raising the possibility of infection of neighboring potato crops. Moreover, several lines that had been released before 1979 were being grown commercially. It was important, therefore, to determine whether PSTV had become established in any of the 1983/1984 certified seed crops. A preliminary survey in early 1983 did not reveal any infection, but this was based on a small number of plants per crop and only a portion of that season's certified seed crops (10).

PSTV cannot be diagnosed reliably by inspecting potato plants because symptoms are often indistinct or absent (2,11). Any survey for such a pathogen requires that samples from many plants be bulked and that a detection method be used that is sufficiently sensitive to detect one infected plant in a bulked sample. The only method that has previously met these requirements is the tomato intermediate/PAGE procedure, in which low concentrations of PSTV are amplified by inoculating tomato plants and subsequently detected by polyacrylamide gel electrophoresis (PAGE) (3,5,12). However, recently described hybridization assays with ^{32}P-deoxyribonucleic acid (DNA) probes have been shown to be much more sensitive than PAGE for detecting viroids (1,6,8), and this raises the possibility of directly testing extracts from bulked potato leaf samples without the need for a tomato intermediate.

In the work described here, we tested the sensitivity of a direct hybridization assay, compared it with a tomato intermediate/hybridization procedure, and used it for a statewide survey of seed potato crops.

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MATERIALS AND METHODS

Collection, subsampling, and bulking of survey samples. Leaves were collected from the potato crops in February and March, 1984 (late summer/early autumn) before the onset of senescence. Sampling was done along evenly spaced transects across rows and, in contrast to our preliminary survey (10), was not biased toward plants with PSTV-like symptoms. Leaflets (three from each plant) were bulked to give 15 20-plant samples (300 plants per crop) and sent to the department's central laboratory at Rydalmere for further processing. Leaf disks 6 mm in diameter were cut from stacks of leaves, further bulked to form 100-plant samples (three per crop), and stored at 5 C for extraction on the following day.

Reference potato leaf samples. To determine the sensitivity of detection methods, reference samples of 200–400 leaf disks were used. PSTV-infected disks were added to noninfected ones in the following proportions: 0/400, 1/400, 2/400, 4/400, and 8/400. One set of reference samples was prepared using first-generation infected plants as the source of infected leaf disks; i.e., plants sprouted from noninfected tuber (cultivar Sebago), inoculated in mid-April with PSTV, and allowed to grow 55 more days at average maximum and minimum temperatures of 30 and 11 C, respectively. A second set was prepared using second-generation plants, plants sprouted from infected tubers in mid-September and grown for 6 wk after the emergence of shoots (average maximum and minimum of 35 and 16 C, respectively). Infected plants were grown in a greenhouse rather than the field to comply with quarantine requirements.

The reference isolate of PSTV was derived from the Glen Innes collection and gave severe symptoms on tomato but indistinct symptoms on potato.

Inoculation and growth of tomato plants. The cotyledons and first true leaf of tomato seedlings (cultivar Grosse Lisse) were dusted with 500-mesh Carborundum and rubbed with a cotton bud soaked with the inoculum. The plants were grown in a greenhouse under controlled-temperature conditions (33 C during the day and 25 C at night), and the terminal leaves were harvested 28–46 days postinoculation.

Extraction of nucleic acids. The following simple procedure was found to give extracts in which PSTV could be detected clearly by the hybridization assay. Bulked potato leaf samples (200–400 fresh leaf disks weighing 0.33–0.66 g) were placed in 50-ml polypropylene centrifuge tubes with 7 ml of phenol mixture (water-saturated phenol containing 0.8% 8-hydroxyquinoline) and 7.5 ml of the buffer (0.5 M sodium acetate-acetic acid, pH 6.0, 10 mM $MgCl_2$, 3% w/v sodium dodecylsulfate, 20%, v/v, ethanol) described by Laulhere and Rozier (4) and homogenized with an IKA Ultra-Turrax tissue disperser. The homogenates were shaken for 10 min at 37 C in a water bath, mixed with 7 ml of chloroform-pentanol (25:1, v/v) at room temperature, and mixed for 10 min more on a platform shaker. The aqueous phase was separated with a bench centrifuge, and the nucleic acids were precipitated with 2.5 volumes of ethanol, kept at −15 C for at least 1 hr,

Table 1. Infectivity of reference extracts on tomato in duplicate inoculation experiments

Proportion of infected potato leaf disks in sample used to prepare extracts	Proportion of extracts giving infection of tomato as shown by hybridization assay	
	First inoculation experiment (June 1984)[a]	Second inoculation experiment (October 1984)
0/400	0/8	0/8
1/400	2/8	1/8
2/400	3/8	4/8
4/400	6/16	9/16
8/400	6/8	7/8

[a]Results from the hybridization experiment of Figure 1.

sedimented at 10,000 *g*, vacuum-dried, resuspended in 1 ml of 0.05 M potassium phosphate buffer, pH 8.0, snap-frozen, and stored at −15 C. Tomato samples were extracted by the same procedure, except two terminal leaflets from each of three replicate-inoculated plants were used (2 g total).

Dot-blot membrane hybridization assay. The hybridization probe, obtained from Biotechnology Research Enterprises of South Australia (BRESA) Pty Ltd., was single-stranded, ^{32}P-labeled cDNA transcribed from M13mp93 vector (1) containing a 195 nucleotide PSTV insert. The dot-blot procedure was exactly as described by Barker et al (1), using 1-μl spots of potato and tomato leaf nucleic acid extracts and 12,000 cpm of probe per square centimeter of nitrocellulose membrane.

RESULTS

Direct hybridization assay. In the dot-blot hybridization assay, extracts containing PSTV give a "hybridization spot" on X-ray film (Fig. 1). Where there were four or eight infected leaf disks in a sample of 400 (4/400, 8/400), intense hybridization spots were observed. The 1/400 and 2/400 dilutions gave spots that were less intense but still clearly distinguishable from the 0/100 control. Leaves from first-generation infected plants (Fig. 1A) gave less intense spots than those of second-generation plants (Fig. 1B), but in both cases, the limit of detection was the same: one infected leaf disk in 400.

An autoradiography period of 6 days was required to reveal the faint spots in Figure 1, but shorter exposures were adequate in another hybridization experiment with a more radioactive probe.

Tomato intermediate/hybridization assay. The potato leaf extracts (first-generation references only) were used to infect tomato plants, and the tomato leaves were tested for PSTV by the hybridization assay. This approach proved less reliable than the direct assay, because many of the reference extracts were not infective, particularly the 1/400 and 2/400 extracts (Fig. 2, Table 1). Results were essentially the same for two inoculation experiments (Table 1).

Direct hybridization assay of survey samples. Sixty-three crops were sampled: 34 crops of cultivar Sebago; eight of Kennebec; five of Kurrel; three each of Sequoia, Exton, and Maramba; two each of Pontiac and Katahdin; and one each of Coliban, Crana, and Early Crana. None of the 100-plant bulked extracts gave a spot when assayed by direct hybridization (Fig. 3). Internal standards (4/400 reference extracts, spotted at the lower right of all four membranes in Figure 3) showed that one infected plant in any of the bulked samples would have been easily detectable. The tomato intermediate/hybridization procedure also gave

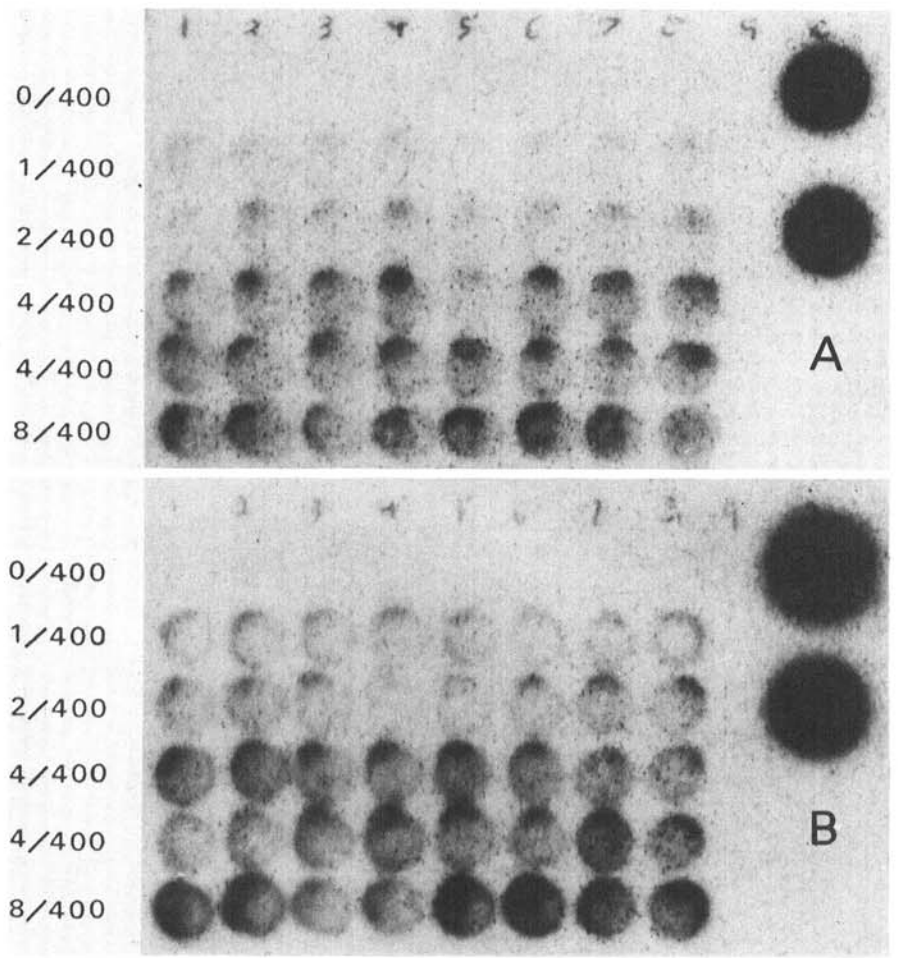

Fig. 1. Test of direct hybridization assay. Each spot represents a single extract. Extracts in each row are replicates prepared by combining disks from different infected potato leaves with noninfected disks. The proportion of infected disks is shown on the left. The two very heavy spots at the far right of each membrane are "100% infected" references. PSTV-infected leaves were obtained from **(A)** first-generation plants inoculated as young sprouts and **(B)** second-generation plants grown from infected tubers. Autoradiography (6 days) and hybridization conditions were identical for membranes in A and B.

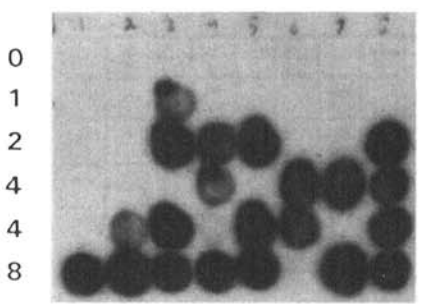

Fig. 2. Test of tomato intermediate/hybridization assay. The potato leaf extracts used as inocula were the same reference extracts that were tested directly in the experiment of Figure 1A, and the number of infected leaf disks in 400 is shown on the left. Each nucleic acid spot was derived from a batch of three tomato plants inoculated with one reference extract. Autoradiography was for 12 hr.

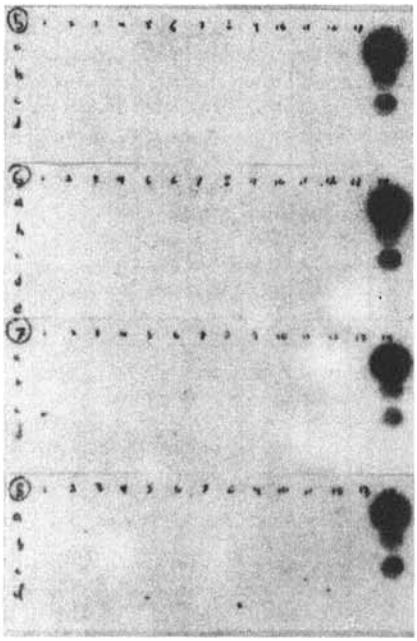

Fig. 3. Direct hybridization assay of survey samples. Extracts of different 100-plant bulked samples fill the entire grid except columns 13 and 14 on all sheets and positions d10, d11, and d12 on the bottom sheet. Reference extracts, spotted at the far right of each membrane (column 14) were 100% infected sample (heavy upper spot) and 4/400 sample (lower two spots).

negative results. Thus, none of the 18,900 plants sampled in the survey was infected with PSTV.

DISCUSSION

The direct hybridization assay proved efficient for conducting a large-scale survey for PSTV. A simple extraction procedure was adequate for preparing potato leaf samples for the assay. We found that two persons working together could subsample and extract leaves from 1,600 plants (16 bulk extracts) in 1 day, and nitrocellulose membranes representing tens of thousands of plants could be analyzed in a single hybridization experiment. Extracts representing a dilution of one infected plant in 100 (4/400 reference samples) gave strong hybridization spots, and even one in 400 was consistently detected. This sensitivity is comparable to that reported for the tomato intermediate/PAGE test (3,5,12).

Leaves from both first- and second-generation plants were detectable by direct assay of bulked samples. These plants were cultivar Sebago infected with a severe isolate and grown in a greenhouse for quarantine reasons. However, we regard them as valid for comparison with field samples for the following reasons: maximum field temperatures in late summer were only slightly less than those recorded in the greenhouse; mild and severe isolates of PSTV have been reported (5,7) to reach similar concentrations in potato leaves; concentrations of PSTV remain fairly constant in potato shoot tips with time after sprouting of infected tubers (5), which suggests that age of the plants is not critical; concentrations of PSTV in different cultivars of potato do not differ more than about threefold (7); and Sebago was the most common cultivar among the crops surveyed. Use of reference infected tissue made it unnecessary to assess sensitivity in terms of micrograms of PSTV.

Contrary to the results of other workers (3,5,12) and our previous experience with extracts of up to six potato plants (10), PSTV was not reliably transmitted to tomato in nucleic acid extracts containing low levels of infected potato leaf tissue. It was therefore unnecessary and undesirable to enhance PSTV concentrations by means of a tomato intermediate. Eliminating this step represents a time savings of at least 4 wk, removes the need for an air-conditioned greenhouse or growth cabinet, and eliminates the risk of cross-contamination between tomato plants.

This was the first report of a field survey aimed at determining the incidence of PSTV in potatoes, although a preliminary survey for potato plants with PSTV symptoms in Canada was reported in 1970 (11). There were no infected plants among the 18,900 plants sampled in the New South Wales survey, which included four cultivars (Kurrel, Maramba, Crana, and Early Crana) that had been bred at Glen Innes and released for commercial use before 1979. On the basis of the binomial distribution theory, the sample size of 300 plants per crop would give a 95% probability of detecting an infection rate of 1%. It therefore appears that PSTV has not become established in certified seed crops and has probably been eradicated in New South Wales. Any suspected occurrences in ware crops or solanaceous weeds could be tested by the survey methods reported in this paper.

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