Rapid Immunological Detection of Potato Viruses in Plant Tissue Squashes

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ABSTRACT

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Squash-blot immunoassay was employed to detect potato viruses X and Y in samples obtained from potato leaves and tubers. Comparative assays were performed by both enzyme-linked immunosorbent assay (ELISA) and squash-blot molecular hybridization. Percentages of agreement between these three methods in leaf samples were 95.0% for potato virus X and 97.2% for potato virus Y. About 0.25 ng of virus per 10 mg of leaf tissue could be detected. The method is comparable with ELISA in terms of simplicity, rapidity, and cost. The procedure was also used to analyze virus distribution in different potato tissues.

Plant viruses are among the principal infectious agents affecting the potato (18). This crop is generally propagated by planting whole tubers or tuber pieces, a method that leads to geographic dissemination of viral diseases and to perpetuation of viral pathogens in tuber stocks. Thus, practices for effective disease control rely heavily on the development of seed potato certification programs. In most developing countries, implementation of these programs is restrained by the absence of adequate facilities and qualified personnel. Even if specialized laboratories are available, massive introduction of routine procedures used in industrialized countries is frequently limited by practical and economical considerations. This is, for example, the case of Argentina, where about 300,000 individual potato samples are examined each year, and where unrestricted use of antibodies and disposable plastic ware are often limiting factors. Clearly, significant improvements in seed quality could be reached if already accepted methods were adapted to meet these local constraints. Besides, the introduction of simpler and inexpensive diagnostic tests will be crucial to establish a domestic seed potato industry in other developing countries in which it does not exist, and where valuable hard currency is invested to purchase certified seed.

Both immunological and molecular hybridization methods have been developed for the most ubiquitous potato viruses (2,7,12). Among immunological methods, enzyme-linked immunosorbent assay (ELISA) is widely used and commonly referred to as a standard technique (5). Other immunological methods, like the dot-blot immunoassay and the tissue printing (4,9,14), were conceived to detect the presence of plant viruses using nylon or nitrocellulose membranes as a solid support. More recently, another procedure, similar to that described in this paper, has been proposed for the specific identification of plant viruses (15). In this work we adapted the squash-blot immunoassay (SBIA) to the routine detection of potato viruses X and Y (PVX and PVY) in leaf and tuber samples from different potato cultivars and compared this technique with other testing procedures.

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MATERIALS AND METHODS

Potato plants. *Solanum tuberosum* L. subsp. *tuberosum* cultivars used in this work (Spunta, Huinkul M.A.G., Ballenera M.A.A., Jaerla, Latina INTA, Diamont, Bright, and Russet Burbank) were obtained from local seed producers. Fifteen to twenty days before planting, tubers were presprouted at 15 C and 90% relative humidity. Plants were grown for 2 mo in a production field located in La Plata, Province of Buenos Aires. At this time, leaves were collected from the upper third of the plants and sampled immediately. Tubers taken from the same plants were collected 2 mo later. Sprouts from these tubers were sampled after a 25-day treatment with the dormancy-breaking compound rindite (10).

Virus strains. PVX (strain CP) and PVY (strain O) were originally provided by the International Potato Center (Lima, Peru). Viruses were propagated in *Nicotiana tabacum* L. and *N. glutinosa* L. by S. F. Nome (Instituto de Fitovirologia, INTA, Córdoba, Argentina) and O. Gracia (EE-INTA, Luján de Cuyo, Argentina) and purified by standard procedures (7,8).

Antibodies. Rabbit anti-PVX and anti-PVY polyclonal antibodies were a gift of M. L. Mayoral (Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela). Both antibodies were resuspended to 1 mg/ml and used at 1:1,000 (v/v) dilutions. Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase (diluted to 0.75 μg/ml) were from Jackson ImmunoResearch (West Grove, PA).

cDNA probes and molecular hybridization. Construction of PVX-CP and PVY-O cDNA genomic libraries were described in previous papers (3,17). Clones comprising the 3′ end region of both viral genomes were chosen as probes. PVX and PVY clones used in this work carry cDNA inserts encompassing 3,555 and 1,182 nucleotides upstream from the respective poly(A) tracts. Double-stranded DNA was obtained by standard plasmid purification (1) and restricted with endonucleases *Eco*RI and *Hin*dIII in order to liberate the inserts. After electrophoresis in agarose gels, cDNA viral fragments were purified by electroelution and radiolabeled with [α-^{32}P]-dATP (New England Nuclear, Wilmington, DE) by the random oligonucleotide priming method (6). Molecular hybridization and processing of squash-blotted samples was conducted as formerly described (11,16). In these conditions, an amount of RNA equivalent to about 1 ng of virions can be detected after an 8-hr autoradiography.

Squash-blotting of potato tissues. Potato tissues were squashed with a hard glass rod onto nitrocellulose and nylon membranes essentially as described by Navot et al (16). Nitrocellulose and nylon membranes BA85 and Nytran were from Schleicher and Schuell (Dassel, Germany) and Nitroplus 2000 from Micron Separations (Westborough, MA). When collecting large numbers of field samples, squashes were performed with the aid of a plastic film template carrying circular holes 0.7 cm in diameter arranged in a regular pattern. In some experiments, tuber slices were dried with a filter paper and then blotted into nitrocellulose membranes with the aid of gentle vacuum. To this aim, nitrocellulose membranes were fit on the filter support of a microsample filtration manifold (Schleicher and Schuell), and tuber slices were slightly pressed over the membranes while vacuum was applied with a water pump for about 10 min.

Immunological detection of PVX and PVY. Membranes with tissue squashes were incubated 1 hr in buffer TBS (50 mM Tris-HCl, pH 8, 150 mM NaCl) containing 0.05% Tween 20, 3% skim milk, and 2% glycine (blocking buffer), and then for another hour in the same buffer containing a 1:1,000 (v/v) dilution

of virus-specific antibodies. Membranes were rinsed three times for 10 min in buffer TBS containing 0.05% Tween 20 and incubated for 1 hr in 10 ml of blocking buffer containing a 1:1,500 (v/v) dilution of goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase. Incubations of membranes (10 × 10 cm) with virus-specific antibodies and conjugates were carried out in sealed plastic bags in a rotatory shaker. Ratio of incubation mix to membrane surface was 100 $\mu l/cm^2$. Membranes were washed for 30 min in buffer TBS and, after a brief rinse in buffer substrate (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$), a color-developing reaction was carried out in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Gibco-BRL, Gaithersburg, MD). All incubation steps were performed at room temperature. Finally, membranes were bleached for 5 min in 1.5% sodium hypochlorite to eliminate color background resulting from plant pigments.

Samples for ELISA tests were collected and processed as previously described (7). Double-antibody sandwich (DAS)-ELISA was conducted, and thresholds of detection established, according to the instructions provided by the manufacturers. Assays were performed in microtiter plates.

RESULTS

Specificity and sensitivity of detection. Whole leaves from potato plants infected with either PVX or PVY were squashed onto nitrocellulose and nylon membranes and probed with antibodies specific for these viruses. As shown in Figure 1, no cross-reaction between PVX- and PVY-infected tissue was detected. Tissues from healthy leaves did not react at all.

In order to estimate the sensitivity of this method, leaf pieces from virus-infected and healthy plants were mixed in different ratios, and 20 mg of the mixtures were squash-blotted with a plastic template as described in Materials and Methods. Dilutions containing known amounts of purified virions of both PVX and PVY were spotted as standards (Fig. 2). To calculate the sensitivity, the signal corresponding to the limit of detection for each virus standard (about 0.1 ng) was equated to the signal corresponding to the limit of detection for infected tissue (about 4 mg of infected tissue). On this basis, a value of about 0.25 ng of virions for every 10 mg of infected tissue was estimated for both viruses. Our current levels of detection by DAS-ELISA are on the order of 5 ng of purified virions (11). Even though all membranes tested gave comparable results, sharper signals were obtained when Nitroplus 2000 membranes were used.

Virus detection in field samples. Eight

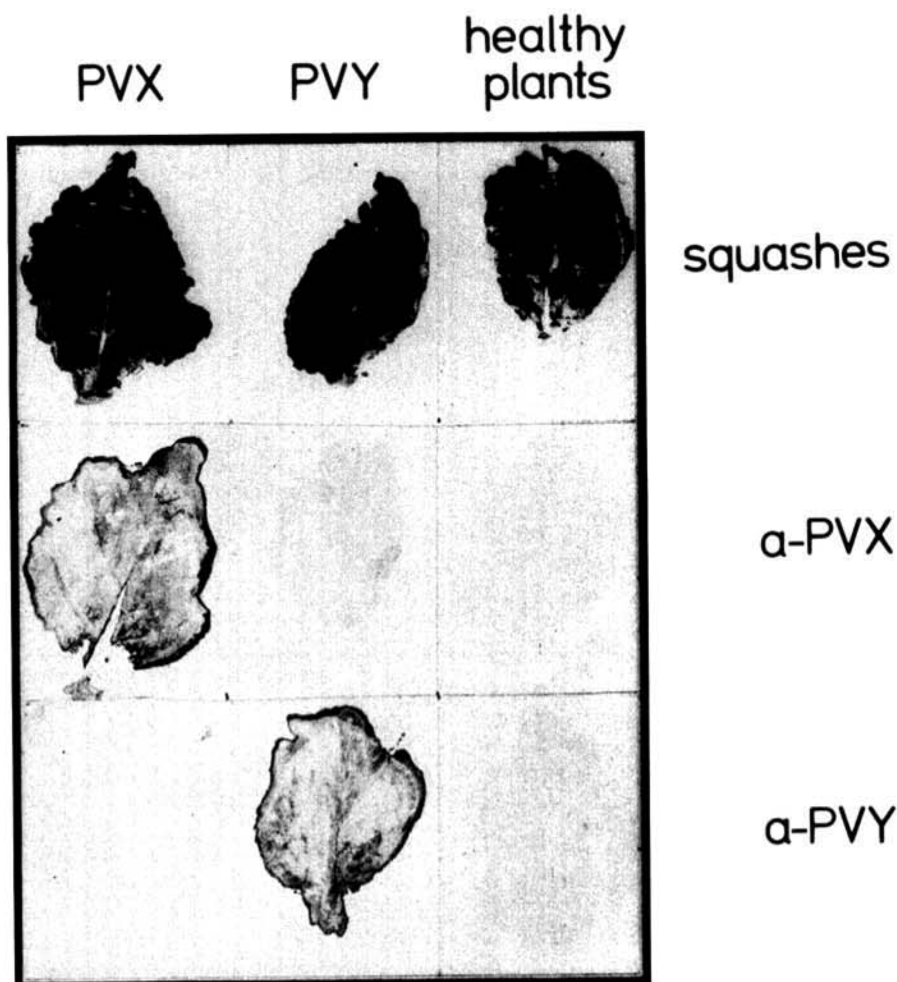

Fig. 1. Specificity of virus detection in leaves from plants infected with potato viruses X and Y (PVX and PVY). Upper row: leaf squash blots from virus-infected and healthy potato plants. Middle row: squash-blot immunoassay (SBIA) of infected and healthy leaf squashes developed with anti-PVX antibodies. Lower row: SBIA of infected and healthy leaf squashes developed with anti-PVY antibodies. Middle and lower row squashes were treated with 1.5% sodium hypochlorite after color development.

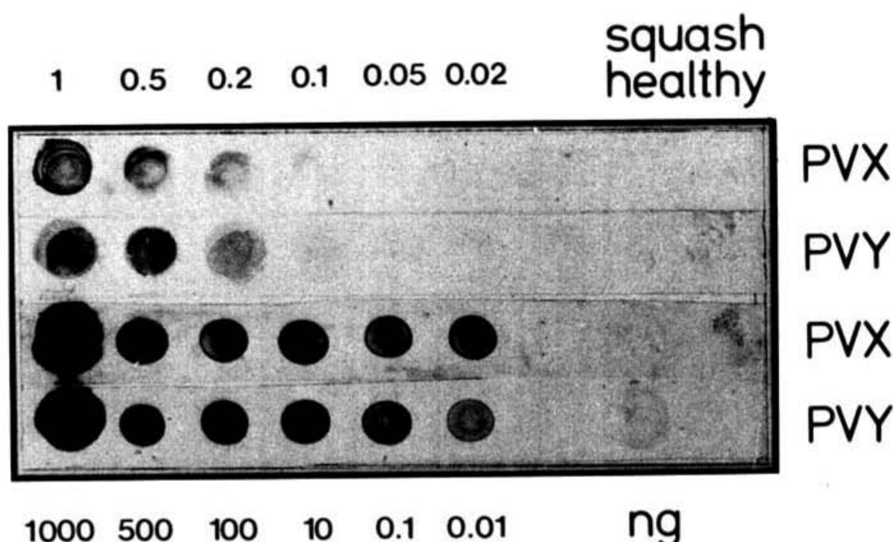

Fig. 2. Levels of detection of potato viruses X and Y (PVX and PVY) in infected potato leaves. Leaf pieces infected with PVX (first line) or PVY (second line) were mixed in different ratios with leaf pieces from healthy plants and squashed into membranes. Ratios of infected to healthy tissue in the mixes are indicated in the upper line. Immunoassays, performed with known amounts of PVY and PVX virions, are included as standards (third and fourth lines). Limit of detection with both virus standards was established at about 0.1 ng. Negative controls were completely colorless after treatment with sodium hypochlorite.

hundred and forty leaf samples of different potato varieties chosen at random from production fields were squash-blotted in duplicate to determine the presence of PVX and PVY. A typical result, showing the detection of PVX in leaves taken from 100 different plants, is depicted in Figure 3. To compare this technique with other well-established methods, single-leaf samples from each of the 181 plants (cultivar Spunta) were examined by SBIA, ELISA, and squash-blot molecular hybridization (SBMH). Agreement obtained by these three methods was 95.0% for PVX and 97.2% for PVY. Similar results were obtained when two lots of tuber sprout samples (95 samples for PVX and 80 samples for PVY, cultivar Russet Burbank) were analyzed in parallel for both SBIA and ELISA. Percentages of concurrence obtained in this case were 95.8 for PVX and 100.0 for PVY.

Several control experiments were carried out to test the feasibility of reutilizing virus-specific antibodies and conjugates. Results showed that immunoreactivity can be preserved without substantial losses after recycling of antibodies for up to five consecutive times. Also, it was found that results were not considerably affected when samples were baked prior to immunological detection (Fig. 4).

Detection of PVX and PVY in different potato tissues. Squashes of leaves, stems, roots, tubers, and sprouts from several infected potato plants were examined for the presence of PVX and PVY. Both viruses were abundantly present in all tissues tested. Signals obtained from squashes of PVX-infected plants are shown in Figure 5A and B. Squashes obtained from roots showed the strongest signals. Similar results were obtained in squashes made to reveal the presence of PVY (*data not shown*). Even though details on viral antigen distribution are suggested in some squashes, sap spreading prevented more precise information. Sharper images were obtained with PVX-infected tubers when blotting was performed under gentle vacuum as described in the methods section. In recently harvested tubers, a higher concentration of viral antigens was evident near the stolon (Fig. 5B, left). Tuber blots made at different stages after breaking of dormancy (Fig. 5B, II and III) showed that viral antigens disseminate first along the cortex and concentrate later in regions close to the sprouts.

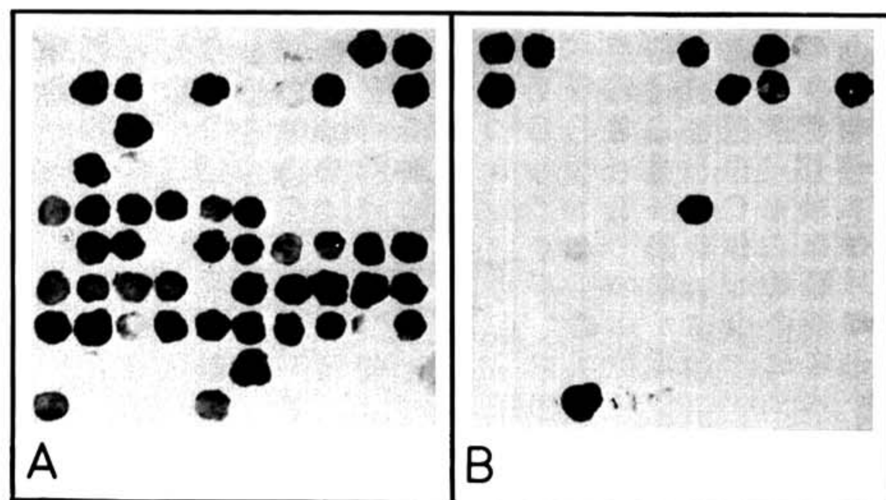

Fig. 3. Squash-blot immunoassay (SBIA) detection of potato viruses X and Y (PVX and PVY) in potato leaf samples collected from the field. A leaf from each of 100 potato plants (Spunta) was squashed in duplicates in Nitroplus 2000 membranes with the aid of a plastic template. Leaves were collected from the upper third of the plants. **(A)** Detection with anti-PVX antibodies. **(B)** Detection with anti-PVY antibodies.

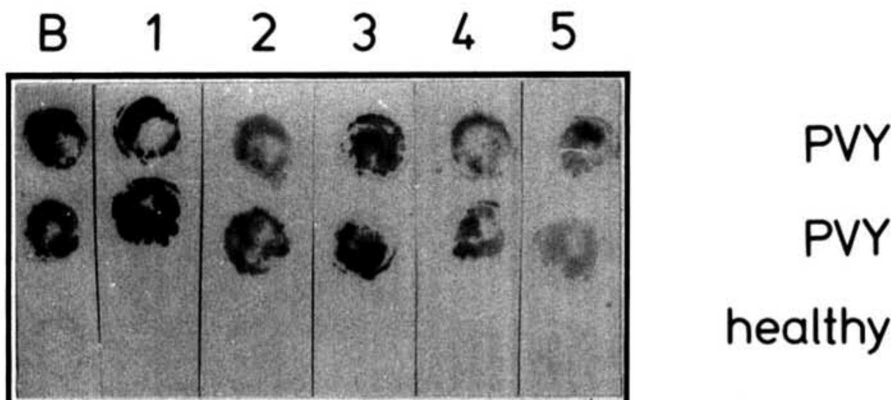

Fig. 4. Effects of baking and reutilization of antibodies and phosphatase conjugates in successive squash-blot immunoassays. Replicates made from potato virus Y (PVY)-infected and noninfected single-leaf potato samples were squashed as described in methods section. Following specific PVY detection on the first replicate, mixes containing antibodies and conjugates were recovered from the incubation bag and kept at 4 C. The same mixes were used to repeat the procedure for five consecutive times. Lanes 1–5, incubation number. Lane B, duplicate of strip number 1, but baked for 2 hr at 80 C before the detection test.

DISCUSSION

The SBIA is a fast and sensitive procedure that could be easily adapted to large-scale indexing of seed potato. In order to evaluate this possibility, this and other detection methods were compared in tests to examine the presence of PVX and PVY in leaf and tuber samples. In agreement with a recent report (13), sensitivity of SBIA was about one order higher than that of routine DAS-ELISA when both methods were compared using virion dilutions. Because of the different extent of virus trapping associated with each sampling procedure (i.e., liquid extraction in ELISA, direct blotting in SBIA), strict quantitative comparisons at the level of tissue samples were difficult to establish. Nevertheless, results from parallel assays performed by SBIA, ELISA, and SBMH indicated that the level of detection of these procedures compare closely in practical terms. Thus, good agreements (95.0% for PVX, 97.2% for PVY) were obtained between these three methods in leaf samples collected from the field. Similar results (95.8% for PVX, 100% for PVY) were obtained in other determinations made by SBIA and ELISA in tuber sprout samples.

In countries with less developed agricultures, advances in seed potato certification programs will primarily arise from the introduction of relatively simple and inexpensive methodologies rather than from major improvements in laboratory facilities or training of personnel. In this connection, several advantages of SBIA are worth mentioning. In contrast to ELISA, SBIA can be performed in large scale without expensive automatic instruments, thus facilitating the introduction of certification tests in poorly equipped laboratories located close to production areas. In addition, squashing requires minimal training and can easily be performed by individual farmers wishing to assess the quality of their own seed potato. In this case, samples could be collected in the field and sent later to distant laboratories for further processing. Since potato viruses are inactivated after baking of the membranes at 80 C, this approach could be employed without risk of virus

dissemination. Other benefits of SBIA related to costs and work efficiency could also be considered. First, incubation into sealed plastic bags reduces the consumption of antibodies and conjugates to about one half of that employed by standard ELISA. Moreover, this incubation procedure allows the recovery of antibodies, a practice that provides additional savings and cannot be routinely implemented when using microwell plates. Second, SBIA competes also with ELISA in terms of working time and simplicity. Assays comprising up to 400 samples can be regularly completed in a single day by a trained operator, and this output can be easily increased if alternative work schemes are adopted. For example, leaf samples can be collected and stocked along several days and then simultaneously processed at the end. Since several hundreds of samples are dealt with together on a single membrane, labor input and manipulations can be greatly reduced at every step.

Finally, the presence of PVX and PVY was detected in squashes of different tissues of infected plants. Even though sap diffusion prevented a detailed view of viral antigen distribution in most tissues tested, clearer images were obtained when tuber samples were blotted under low vacuum. These results suggest that SBIA could be further developed as a very useful technique to monitor virus spreading during potato infections.

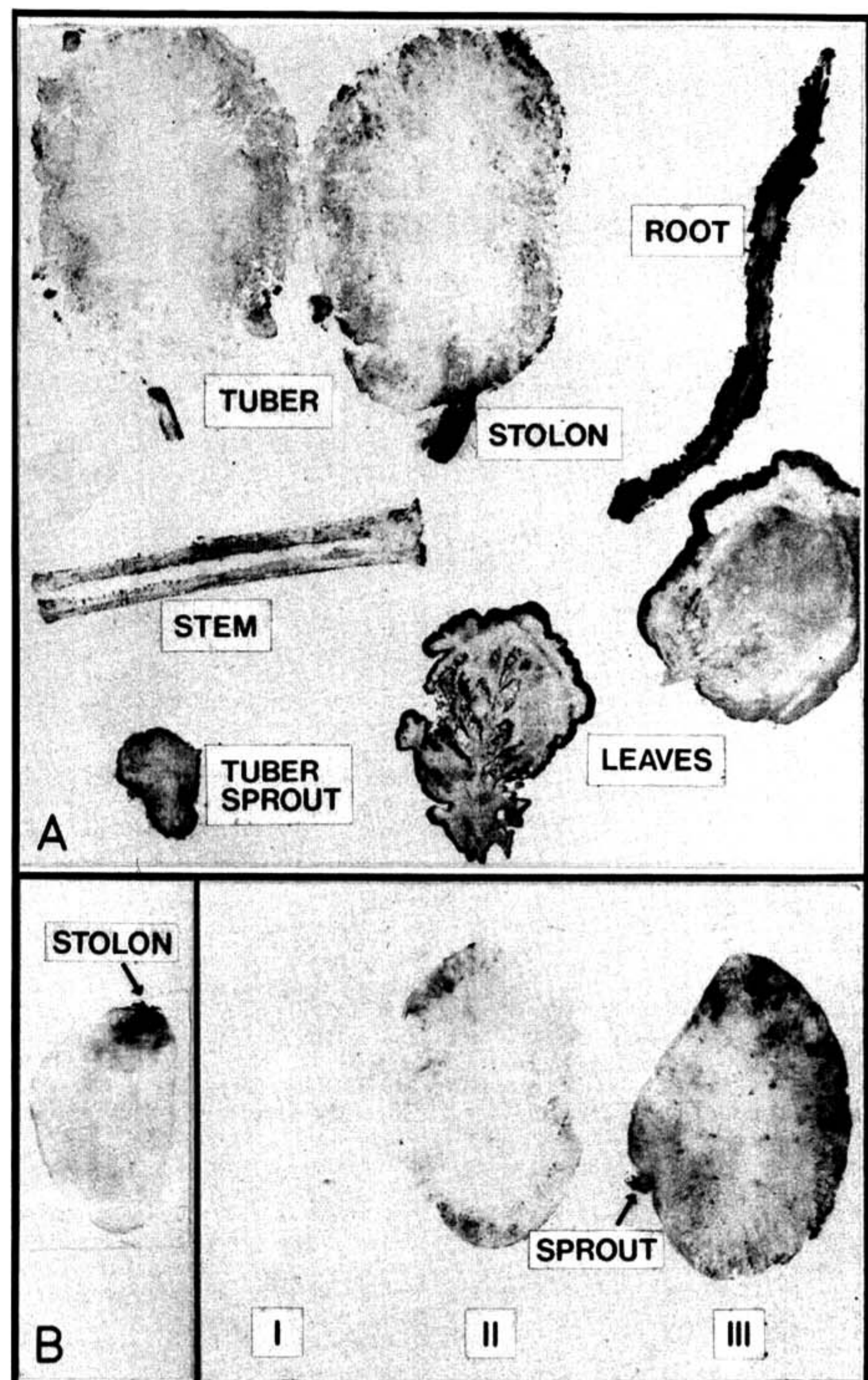

Fig. 5. Squash-blot immunoassay of potato virus X (PVX) in squashes of different potato tissues. **(A)** Different tissues from an infected potato plant (Spunta) were blotted into Nitroplus 2000 membranes and assayed for the presence of PVX as described in methods section. Tissues from five individual plants were examined; results shown correspond to a single individual. **(B)** Tuber slices (Spunta) were blotted under low vacuum as described in methods section. Left, infected tuber sampled 1 wk postharvest; right (I-III), tubers kept in the dark and sampled 10 wk postharvest. I, healthy potato tuber; II, nonsprouted infected tuber; III, infected tuber showing visible sprouts. Tubers from 10 individual plants were examined. Images shown in the figure were selected from different individuals.

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LITERATURE CITED

1. Birmboim, H. C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. 100:243-270.
2. Boulton, R. E., Jellis, G. J., Baulcombe, D. C., and Squire, A. M. 1986. The application of complementary DNA probes to routine virus detection, with particular reference to potato viruses. Pages 41-53 in: Developments and Applications in Virus Testing. R. A. C. Jones and L. Torrance, eds. Lavenham Press, Lavenham, Sudbury, England.
3. Bravo-Almonacid, F., and Mentaberry, A. 1989. Nucleotide cDNA sequence coding for the PVY coat protein. Nucleic Acids Res. 17:401.
4. Cassab, G. I., and Varner, J. E. 1987. Immunocytolocalization of extensin in developing soybean seed coats by immunogoldsilver staining and by tissue printing on nitrocellulose paper. J. Cell Biol. 105:2581-2588.
5. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunoabsorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
6. Feimberg, A., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.
7. Gugerli, P. 1978. The detection of two potato viruses by enzyme-linked immunoabsorbent assay (ELISA). Phytopathol. Z. 92:51-56.
8. Gugerli, P. 1979. Le test immuno-enzimatique (ELISA) et son application pour le diagnostic rapide des virus de la pomme de terre. Rev. Suisse Agric. 11:253-260.
9. Hawkes, R., Niday, E., and Gordon J. 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. 119:142-147.
10. Hiddema, J. 1972. Inspection and quality grading of seed potatoes. Pages 206-215 in: Viruses of Potato and Seed Potato Production. J. A. De Bokx, ed. Pudoc, Wageningen, Netherlands.
11. Hopp, H. E., Giavedoni, L., Mandel, M. A., Arese, A., Orman, B., Bravo-Almonacid, F., Torres, H. N., and Mentaberry, A. N. 1988.

Biotynilated nucleic acid hybridization probes for potato virus detection. Arch. Virol. 103:231-241.
12. Hopp, H. E., Haim, L., Bravo Almonacid, F., Tozzini, A. C., Orman, B., Arese, A. I., Ceriani, M. F., Saladrigas, V., Celnik, R., del Vas, M., and Mentaberry, A. 1991. Development and application of a non radioactive nucleic acid hybridization system for simultaneous detection of four potato pathogens. J. Virol. Methods 31:11-30.
13. Hsu, H. T., and Lawson, R. H. 1991. Direct tissue blotting for detection of tomato spotted wilt virus in *Impatiens*. Plant Dis. 75:292-295.
14. Koening, R., and Burgermeister, W. 1986. Applications of immunoblotting in plant virus diagnosis. Pages 121-137 in: Developments and Applications in Virus Testing. R. A. C. Jones and L. Torrance, eds. Lavenham Press, Lavenham, Sudbury, England.
15. Lin, N. S., Hsu, Y. H., and Hsu, H. T. 1990. Immunological detection of plant viruses and a mycoplasmalike organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80:824-828.
16. Navot, N., Ber, R., and Czosnek, H. 1989. Rapid detection of tomato yellow leaf curl virus in squashes of plants and insect vectors. Phytopathology 78:562-568.
17. Orman, B., Celnik, R. M., Mandel, A. M., Torres, H. N., and Mentaberry, A. N. 1990. Complete cDNA sequence of South American isolate of potato virus X. Virus Res. 16:293-306.
18. Rich, A. E. 1983. Potato Diseases. Academic Press, New York.