A Radial-Immunodiffusion Test for the Simultaneous Diagnosis of Potato Viruses S and X

James F. Shepard

Department of Botany and Microbiology, Montana State University, Bozeman 59715.
The technical assistance of G. Secor and J. Morton is gratefully acknowledged.
Montana Agricultural Experiment Station Journal Series Paper No. 168.
Accepted for publication 19 June 1970.

ABSTRACT

The single-radial-diffusion procedure was investigated as a possible means of detecting potato virus S (PVS) as well as potato virus X (PVX) in infected plants. PVS and PVX could both be degraded with 30% pyridine. Pyridine-degraded PVS protein was only useful as immunizing antigen, however, for it would not diffuse well through agar gels. The degradation of purified PVS and PVX with 2.5% pyrrolidine, in contrast, resulted in the production of rapidly diffusing antigen in the case of both viruses. Pyrrolidine was found to be similarly effective for the testing of infective leaf saps when PVS- and/or PVX-degraded protein (D-protein) antisera were impregnated in agar gels. Additional studies with numerous strains of PVX indicated that the single-radial-diffusion procedure for the detection of PVX D-protein fragments should not be restricted to the strain used for immunization. Phytopathology 60:1669-1671.

Additional key words: serology, virus degradation.

A rapid yet more reliable means of diagnosing potato virus S (PVS) infections on a mass scale is desirable for use in virus-free potato programs. Not only is an appropriate indicator host lacking for this virus, but PVS is normally in lower concn in the potato plant. The dependence of PVS distribution upon variety and stage of plant development further complicates the diagnosis (1). Thus, the sensitivity of a serological test should be sufficient to detect μg amt of antigen if it is to be at all reliable for routine PVS diagnosis. The recently described procedure for potato virus X (PVX) serodiagnosis (the single-radial-immunodiffusion test) (4) seemed to be of potential value for PVS. As with PVX, the use of the radial-diffusion procedure for PVS would necessarily be dependent upon the fragmentation of the virus into serologically active low mol-wt oligomers. PVS is too elongate to diffuse adequately through agar gels.

This paper reports the application of the single-radial-diffusion test to the serological detection of both PVS and PVX with the same degradative procedure.

Materials and Methods.—*Potato virus X.*—Potato virus X (PVX) was purified and degraded-protein (D-protein) antiserum prepared as previously described (4). Numerous additional strains were also used as test antigens. Most strains were isolated from different potato cultivars by serial single lesion transfers on *Gomphrena globosa* L. Single lesion isolates were inoculated onto tobacco (*Nicotiana tabacum* L. 'White Burley') which under constant environmental conditions was a suitable strain-differentiating host.

Potato virus S.—Potato virus S (PVS) was purified from a singly infected clone of potato (*Solanum tuberosum* L. 'Norgold Russet'). Where appropriate, infectivity assays were conducted on *Nicotiana debenyi* Domin. Infected foliage was homogenized with a blender in 0.05 M borate buffer pH 8.2 containing 1% sodium sulfite. Two ml of buffer were used/1 g of tissue. Homogenates were squeezed through cheesecloth and clarified by chloroform emulsification and slow-speed centrifugation (10 min at 10,000 *g*). The aq phase was centrifuged for 90 min at 78,000 *g*, and the sedimented virus resuspended in 0.05 M Na citrate buffer pH 6.5. Resuspended PVS was centrifuged at low speed and the virus then precipitated from the supernatant suspension with 5% (final) polyethylene glycol (PEG, mol wt 6,000) (2). Viral precipitates were sedimented at low speed, and after decantation of supernatant solutions, they were resolubilized in 0.05 M citrate buffer. Further purification was achieved by two additional cycles of differential ultracentrifugation. The purity of PVS prepared in this manner was verified by water-clear final pellets, by the absence of ribosome-like particles when preparations were viewed with an electron microscope, and by the absence of host reactive antibody in PVS-degraded protein antisera.

For use as immunizing antigen, PVS was adjusted to a concn of 2-3 mg/ml prior to degradation. PVS concn were determined spectrophotometrically using an extinction coefficient of 3.0 (R. Stace-Smith & J. Tremaine, *personal communication*). PVS was degraded by the addition of either 30% pyridine or 2.5% pyrrolidine (final concn). Either of these organic compounds was suitable for the preparation of PVS-degraded protein (D-protein) immunogen. Fragmented virus was then dialyzed for 24 hr against 0.05 M borate buffer pH 8.2 containing 0.37% formaldehyde. In consideration of previous results with PVX D-protein, formaldehyde stabilization of PVS D-protein for immunization was used (5). PVS D-protein antisera were produced in rabbits by the same administration procedure as for PVX D-protein antisera.

Serology.—Antiserum titers to PVX- and PVS-degraded proteins were evaluated in 100-mm double-immunodiffusion plates containing 10 ml of 1% Ionagar No. 2 (Colab Inc.) dissolved in 0.05 M Tris[tris(hydroxymethyl) aminomethane]-HCl buffered saline. PVS D-protein antiserum titers were determined with pyrrolidine-degraded PVS protein at 1 mg/ml as the test antigen, and PVX D-protein serum titers ascertained as before (4).

Single radial-diffusion plates were prepared as pre-

viously reported (4), except that Ionagar was used as the diffusion medium and not purified agar (Difco). Stock solutions of Ionagar were at a concn of 2% in 0.05 M Tris-saline buffer, pH 7.2. Agar was mixed at 50 C with an equal volume of antiserum diluted with the same buffer. Anti-PVX D-protein and anti-PVS D-protein radial diffusion plates contained 2.5 and 3 ml of antiserum (titer = 1/16), respectively.

RESULTS.—*Antisera.*—PVX and PVS D-protein antisera routinely displayed homologous precipitin titers of 1/16 or 1/32 after a 4-week immunization period. In the case of PVS, D-protein antiserum titers to D-protein prepared with pyrrolidine were the same whether pyridine or pyrrolidine was used to prepare the immunizing antigen.

Tests of degrading agents with purified virus.—Pyridine is an effective compound for the degradation of PVX either for immunization or serological testing. The addition of 30% pyridine to PVX preparations resulted in an immediate loss of opalescence and stream birefringence, and the formation of rapidly diffusing antigen. When pyridine at the same concn was mixed with PVS preparations, a similar disappearance of opalescence and birefringence occurred; however, when tested in double-immunodiffusion against antiserum prepared against the same antigen, pyridine-degraded PVS preparations produced extremely faint or no reactions. Tube precipitin tests with the same combination, in contrast, resulted in copious precipitates. It appeared from this that pyridine-degraded PVS protein was effective for antiserum production, but was not readily diffusible in agar gels. Similar protein preparations in radial-diffusion systems containing PVS D-protein antiserum produced faint precipitin rings close to the periphery of the depot. This contrasted sharply with the normal behavior of degraded PVX.

Other compounds were tested in radial- and double-diffusion in an attempt at improving the diffusibility of PVS-degraded protein. Briefly, these included final concn of 1% sodium dibutylnaphthalenesulfonate (Leonil SA), 1% sodium dodecyl sulfate (SDS), 1 M guanidine hydrochloride, 0.1 N NaOH, and 0.5 M glycine buffer, pH 12.0. Of these, only glycine buffer pH 12 consistently produced diffusible antigenic fragments when mixed with purified PVS. The efficiency of 0.5 M glycine degradation, however, was questionable. When PVS and PVX at the same concn were mixed with glycine buffer and separately tested in radial-immunodiffusion plates, PVX precipitin rings were always much more intense and larger in diam than with PVS.

Another organic compound which is readily soluble in water is pyrrolidine. Pyrrolidine, unlike pyridine, is strongly basic. When a final concn of 2.5% pyrrolidine was added to a PVS preparation and the mixture tested in either radial- or double-diffusion systems against PVS D-protein antiserum, intense precipitin reactions were observed. Similar reactions did not occur on normal serum control plates or with concd protein preparations from uninfected potato plants. Experiments with pyrrolidine at the same concn with PVX preparations

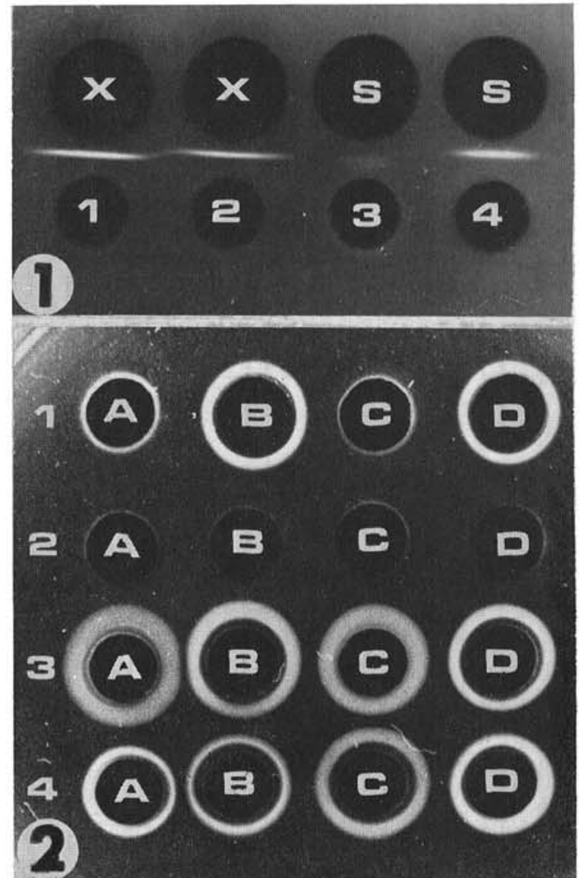

Fig. 1-2. **1)** A double-diffusion system with potato virus X- and potato virus S-degraded protein against PVX D-protein antiserum (wells labeled X) and PVS D-protein antiserum (wells labeled S). Depots 1 and 2 were charged with pyridine- and pyrrolidine-degraded PVX, respectively. Depots 3 and 4 were filled with PVS degraded with the same respective compounds. **2)** Tests on a single-radial-diffusion plate containing mixed PVS and PVX D-protein antisera. Row 1 across consists of depots containing purified PVS degraded with 30% pyridine (well A) and 2.5% pyrrolidine (B); and PVS infective potato leaf saps treated with pyridine (C) and pyrrolidine (D). Row 2 contains similarly treated protein preparations (depots A and B) and crude extracts (C and D) from uninfected potatoes. The depots in row 3 contain PVX preparations and crude extracts exposed to the same respective reagents as rows 1 and 2. In row 4 are pyridine-treated leaf extracts containing the B strain (A), a latent strain (B), a ringspot strain (C), and the standard mottle strain (D) of PVX.

yielded similar results against PVX D-protein antiserum.

Thus, for purified preparations, pyrrolidine at a final concn of 2.5% appeared to be a useful, degrading agent for both PVX and PVS (Fig. 1).

Experiments with leaf sap.—Pyridine (30%) and pyrrolidine (2.5%) were tested with foliar extracts from potatoes separately infected with PVS and PVX and sap from uninfected potatoes. Either pyridine or pyrrolidine was mixed with extracts, and mixtures were

then compared in radial-diffusion plates against homologous and normal serum. Pyridine and pyrrolidine were found to be equally effective degrading agents for the detection of PVX in infected leaf extracts. Precipitin rings were of similar diam and intensity with both compounds (Fig. 2, row 3). For PVS, however, pyridine was of little value. Treatment of infective saps with pyridine failed to result in diffusible antigen reactive with PVS D-protein antiserum. In contrast, pyrrolidine at a final concn of 2.5% was found to be quite useful. Infective leaf extracts routinely exhibited specific precipitin rings in radial-diffusion when premixed with pyrrolidine (Fig. 2, row 1). Thus, a final concn of 2.5% pyrrolidine was effective for the production of diffusible antigenic fragments in sap from either PVX- or PVS-infected plants.

Detection of PVS and/or PVX on the same radial-diffusion plate.—PVX D-protein antiserum (2.5 ml) was combined with 3 ml of PVS D-protein antiserum and mixed with 4.5 ml of buffered 2% Ionagar. After gelation, depots were punched with a No. 1 cork borer. The depots were then charged individually with pyridine- and pyrrolidine-treated PVX- and PVS-purified preparations and infective leaf extracts. The results indicated that mixed PVX and PVS D-protein antisera provided the same results for the respective virus as with either antiserum alone. Furthermore, either PVS or PVX or both could be reliably detected on the same radial-diffusion plate if 2.5% pyrrolidine was the degrading agent used (Fig. 2, rows 1 and 3). If on the other hand, pyridine was employed, only PVX could be diagnosed.

Use of PVX D-protein antiserum for the detection of other PVX strains.—Numerous strains of PVX were kindly provided for this investigation. These included the B-strain of PVX (PVX-B) by D. Govier, Rothamsted Experiment Station; a ringspot strain by C. Knight, University of California, Berkeley; and latent, mild, and severe strains from the Shultz collection by R. Goth, USDA, Beltsville, Md. In addition, other isolates deviating in virulence on White Burley tobacco from that of the standard strain were investigated.

Infective leaf sap or partially purified preparations of all of the isolates collected were compared in double-diffusion after pyridine degradation with similarly treated extracts or preparations of the standard PVX strain. The results indicated that on the basis of the degraded protein antigen, none of the PVX isolates tested, with the exception of PVX-B, could be serologically differentiated with D-protein antiserum. In radial-diffusion plates, strong precipitin rings developed in the case of all strains including PVX-B (Fig. 2, row 4). A more detailed study of the antigenic relationship between the degraded proteins of PVX-B and the standard PVX isolate will be the subject of another report.

Discussion.—The use of chemically degraded PVS fragments as test antigens in single-radial-diffusion systems should be a reliable diagnostic procedure for this virus as well as for PVX. Since pyrrolidine is an effective degrading agent for both PVS and PVX, the presence of either virus in a plant may be verified on a single test plate containing mixed antisera; however, existing knowledge as to the behavior of PVS in host plants should be considered. For example, the concn of PVS has been suggested to increase significantly with age of the plant. Hence, serological testing is more reliable if older plants are indexed (1).

It is of interest that pyridine and certain other degrading compounds are unsuitable for PVS testing even though some of these would produce useful immunizing antigen. The detergents tested, Leonil SA and SDS, were especially unsuitable for radial-diffusion testing because of the all too frequent spurious reactivity. The suitability of pyrrolidine for either PVS or PVX points out the potential of organic compounds other than detergents and some salts as effective degrading agents for serodiagnosis. A wide range of possible degrading compounds would be desirable, for, as exemplified with PVS, a single reagent most probably will not be useful for all viruses. Even though pyrrolidine is strongly basic, the pH factor alone cannot account for its activity of PVS. Glycine buffer at pH 12.0 was but nominally effective, and 0.1 N NaOH ineffective, for the production of diffusible antigenic fragments.

From the results of the PVX strains investigated, it does not appear that the use of PVX D-protein antiserum for serodiagnosis must be restricted to those strains which are identical either in virulence or antigenic specificity. It has been demonstrated (3) that the serological reactivity of a PVX strain is not a function of its virulence. Furthermore, most PVX variants are either serologically identical or at least closely related. Of the strains investigated by Matthews (3), PVX-B differed from the general class of isolates in antigenic specificity to as great a degree as any. In the present study, PVX-B-degraded protein was readily detectable with antiserum to the D-protein of the standard isolate. It is likely that a similar condition may also be true for strains of PVS.

LITERATURE CITED

1. European and Mediterranean Plant Protection Organization. 1963. Appendix VII. Methods of diagnosis of potato virus diseases in the Soviet Union. Potato Virus Diagnosis, Series A. 91 p.
2. Gooding, G. V., & T. T. Hebert. 1967. A simple technique for purification of tobacco mosaic virus in large quantities. Phytopathology 57:1285.
3. Matthews, R. E. F. 1948. Studies of potato virus X. II. Criteria of relationship between strains. Ann. Appl. Biol. 36:460-474.
4. Shepard, J. F., & G. A. Secor. 1969. Detection of potato virus X in infected plant tissue by radial and double-diffusion tests in agar. Phytopathology 59:1838-1844.
5. Shepard, J. F., & T. A. Shalla. An antigenic analysis of potato virus X and its degraded protein. I. Evidence for—and degree of—antigenic disparity. Virology (in press).