Detection of Potato Viruses S, X, and Y by Enzyme-Linked Immunosorbent Assay on Nitrocellulose Membranes (Dot-ELISA)

E. E. BANTTARI, Professor, and P. H. GOODWIN, Research Assistant, Department of Plant Pathology, University of Minnesota, St. Paul 55108

ABSTRACT

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Potato viruses S, X, and Y were detected by enzyme-linked immunosorbent assay (ELISA) on nitrocellulose membranes at dilutions of 1:16,000, 1:128,000, and 1:16,000, respectively (parts infected potato sap:parts healthy potato sap). The assay for potato virus X was at least eight times more sensitive than double-antibody sandwich ELISA (DAS-ELISA) in polystyrene cuvettes. DAS-ELISA was used with alkaline phosphatase conjugates of each gamma globulin and a 0.45-μm pore size nitrocellulose membrane was used as the solid phase. Naphthol AS-MX phosphate and fast red TR salt were used as the substrate and stain that produced visually detectable "dots" on the membranes. Quantitative data on reaction intensities were obtained with a reflectance densitometer. This ELISA modification is termed dot-ELISA.

Additional key words: immunoblot assay

Although polystyrene plastics in the form of microtiter plates, cuvettes, or beads have most commonly been used in enzyme-linked immunosorbent assays (ELISA), other materials such as polyvinyl tubes (4), cyanogen bromide-activated paper (8), and nitrocellulose membranes (NCM) (3,6,7,9,10–12,15) have also been used to bind antibodies and other proteins. Rybicki and Von Wechmar (12) degraded several tobamoviruses and bromoviruses, electroblotted the subunit proteins to NCM, and probed them with virus-specific rabbit antisera. The virus proteins were indirectly detected with horseradish peroxidase-conjugated antirabbit globulins.

In some applications, NCM were employed as the solid phase in indirect ELISA in which antigen was electrophoretically transferred or "blotted" to the NCM. After incubating and washing the antigen, a blocking agent was added to saturate any unoccupied binding sites on the NCM. Then IgG rabbit antibody specific to the antigen to be detected was incubated with the NCM, and unbound antibody was removed by washing. Horseradish peroxidase-conjugated antirabbit second IgG was added, incubated, and washed to remove unbound antibody. Finally, a histochemical substrate was added that was hydrolyzed to form water-insoluble colored spots or dots on the membrane. Horseradish peroxidase, the most frequently used enzyme, produced purple spots after addition of substrate. The membranes, however, had to be be stored in the dark to prevent fading of the spots (1). Alkaline phosphatase reacts with naphthol AS-MX phosphate mixed with 5-chloro-2-toluidinediazonium chloride hemizene chloride (fast red TR salt) to produce red dots or with diazotized 4-benzolamino-2,5-dimethoxyaniline $ZnCl_2$ (fast blue BBN salt) to produce blue dots on the white background of the NCM.

As little as 50 pg of antigen or 100 pg of antibody (1) were detected using horseradish peroxidase and 4-chloro-1 naphthol, *o*-phenylenediamine, *O*-dianisidine, or 3,3-diamino-benzidine substrates. Densitometric analyses using the reflectance of the colored spots have been used to provide quantitative measurements of reaction intensities (7). The experiments reported here were made to test the applicability of double-antibody sandwich (DAS-ELISA) on NCM for potato viruses S, X, and Y (PVS, PVX, and PVY). Several applications of ELISA to detect antigens on NCM have been termed dot-blot, immunoblot, and electroblot ELISA. For convenience, we have chosen to call our assay dot-ELISA.

MATERIALS AND METHODS

PVS, PVX, and PVY were maintained in the greenhouse and in the field in *Solanum tuberosum* L. 'Norland,' 'Kennebec,' and 'Russet Burbank.' Greenhouse-grown *Nicotiana tabacum* L. 'White Burley' was also used as a host for PVY. Healthy plants of each potato cultivar or tobacco were used for healthy controls and to make virus dilutions.

The gamma globulins and enzyme conjugates for each virus were prepared according to the techniques of Clark and Adams (5), except globulin fractions were not passed through DE cellulose.

Dot-ELISA. Several techniques were tested for ELISA on NCM, but the procedure selected for most experiments was a modification of DAS-ELISA (5), using 0.45-μm Millipore HAHY or HAWP NCM filters for the solid phase. Each NCM was wetted by floating it on distilled water for a few seconds, then transferred with forceps to a plastic petri dish containing coating gamma globulin (first antibody) in bicarbonate buffer, pH 9.6. Coating gamma globulin for each antiserum was tested at concentrations of 0.1, 1, and 10 μg/ml of coating buffer. The 1-μg/ml concentration produced the lowest virus-free and highest virus-specific dots and was chosen for both dot-ELISA and DAS-ELISA. The NCM were incubated for 4 hr in coating gamma globulin, then rinsed five times within 30

Present address of second author: Department of Plant Pathology, University of California, Davis 95616.

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min with 10 mM Tris-HCl (Sigma Chemical Co., St. Louis, MO) buffer, pH 7.4, plus 0.9% NaCl plus 0.05% Tween 80 (Sigma) (TBS-T80). A blocking agent consisting of TBS-T80 plus 3% bovine serum albumin (BSA) (Sigma) was added to saturate any unoccupied binding sites on the NCM. After incubation with blocking agent for 1.5–2 hr at 36 C, the NCM was used directly without further washing for deposition of antigen. Plastic templates were used to confine plant sap in wells on the NCM. Several sizes of templates (3–12 mm thick) with drilled 4–8 mm holes to form 14–150 wells were tested (Fig. 1). The bottom half of each template contained holes that corresponded to holes in the upper half. Templates with 5-mm-diameter wells held about 400 μl of sap plus additive. A Whatman No. 1 filter paper cut to the size of the template was saturated with TBS-T80 buffer and placed over the bottom half of the template. The gamma globulin-coated NCM was taken directly out of the blocking agent, placed on the filter paper, and covered with the top half of the plastic template, which was bolted tightly to the bottom. For experiments with PVS and PVY, duplicate wells were filled (400 μl) with antigen diluted 1:1 with TBS-T80 plus 0.01 M sodium diethyldithiocarbamate (NaDIECA) (Matheson, Coleman and Bell, Norwood, OH). For PVX assays, 12.0% Na_2SO_3 was added to TBS-T80 to dilute the antigen 1:1. The template was placed in a moist box to prevent drying and kept at 4–5 C overnight. The next morning, the template was disassembled and the NCM washed vigorously with deionized water to remove plant debris from the dots. Usually, the debris washed away readily, leaving only pale yellow or slightly greenish spots where sap had been deposited. The NCM was transferred to a plastic petri dish and washed five times within 30 min with TBS-T80. Alkaline phosphatase (Sigma) conjugated to virus-specific gamma globulin (1:800 dilution) was added to each petri dish to immerse the NCM, which was then incubated at room temperature for 3–4 hr. The enzyme conjugates were decanted and saved for reuse, and the NCM were washed five times within 30 min with TBS-T80.

Substrate was prepared by dissolving 6 mg/ml of one of the salts, fast red TR salt, fast blue BBN salt, or 5-chloro-4 benzamido-2 methylbenzene-diazonium chloride (fast red-violet LB salt) (Sigma) in 0.2 M Tris-HCl buffer, pH 8.2. The solutions were filtered through Whatman No. 1 filter paper and mixed 1:1 with naphthol AS-MX phosphate (Sigma) (diluted to 0.1% in 0.2 M Tris-HCl buffer, pH 8.2). The substrate was then added to the NCM. The enzymatic reaction was terminated within 0.5–1 hr by rinsing the NCM in deionized water, and the membranes were air-dried for permanent storage. Positive reactions were differentiated visually or by reflectance measurements using a MacBeth TR-524 reflectance densitometer (MacBeth Color and Photometry Division, Kollmorgen Corp., Newburgh NY). Positive readings were those in which the mean of the virus-specific reflectances exceeded the mean of the healthy sap background reflectance plus two standard deviations of the mean of the healthy sap.

DAS-ELISA in polystyrene cuvettes. DAS-ELISA was used as a comparison of efficacy of dot-ELISA. Preparation and washing of cuvettes and absorbance measurements were done with a Gilford PR-50 EIA processor analyser (Gilford Instruments, Inc., Oberlin, OH) as described previously (2). Identical samples were diluted and used for both DAS- and dot-ELLSA. For DAS-ELISA, 0.1 ml of 0.01 M NaDIECA in phosphate-buffered saline plus 0.05% Tween 20 (PBS-T) was added to each well with 0.1 ml of diluted PVS- or PVY-infected sap. For PVX, 0.1 ml of 12.0% sodium sulfite in PBS-T was mixed with 0.1 ml of PVX dilution and deposited in each well. Positive reactions were those in which the mean of the virus-specific absorbances (A_{405}) exceeded the mean of healthy sap absorbances plus two standard deviations of the healthy mean.

RESULTS

Dot-ELISA. In developing the dot-ELISA technique, several types of NCM with different pore sizes were compared. Millipore GSWP, HAWP, or HAHY filters (0.22-, 0.45-, 0.8-, and 1.2-μm pore sizes) were tested. Filters with pore sizes of 0.45–1.2 μm all developed about equal color intensities for PVX and PVY. However, the 0.45-μm filters were less fragile than the larger pore size filters and permitted better drainage than the 0.22-μm pore size. They were used in all subsequent experiments. Hydrophilic Durapore GVWP (0.22-μm pore size), hydrophobic Durapore HVHP (0.45-μm pore size), and Zeta-Probe (Bio-Rad Laboratories, Richmond, CA) were also tested but produced either high background color or low virus-specific reactions. Several blocking agents including 3% BSA, 0.6% ovalbumin (0), or 0.25% gelatin were tested for their ability to saturate unoccupied binding sites on gamma globulin-coated NCM. Ovalbumin and BSA were both effective, and 3% BSA in TBS-T80 was selected as the blocking agent in most experiments.

Three histochemical stains (fast red TR, fast blue BBN, and fast red-violet LB) were compared; all three produced bright colors that contrasted sharply with the white NCM. Color intensities were measured with a MacBeth densitometer. The highest reflectances were obtained for fast blue BBN stain with a neutral filter and fast red TR with a magenta filter. The magenta filter with fast red TR stain was used in all subsequent experiments in which reflectance measurements were made.

Dot-ELISA vs. DAS-ELISA for detecting PVS, PVX, and PVY in greenhouse-grown plants. Assays to compare dot- with DAS-ELISA for detection of PVS, PVX, and PVY were made from plants grown in the greenhouse in the winter and spring (Table 1). PVX was detected in sap dilutions of at least 1:128,000 (Fig. 2), whereas the dilution end point of PVX was 1:16,000 by DAS-ELISA. PVS and PVY were detected by dot-ELISA at a 1:64,000

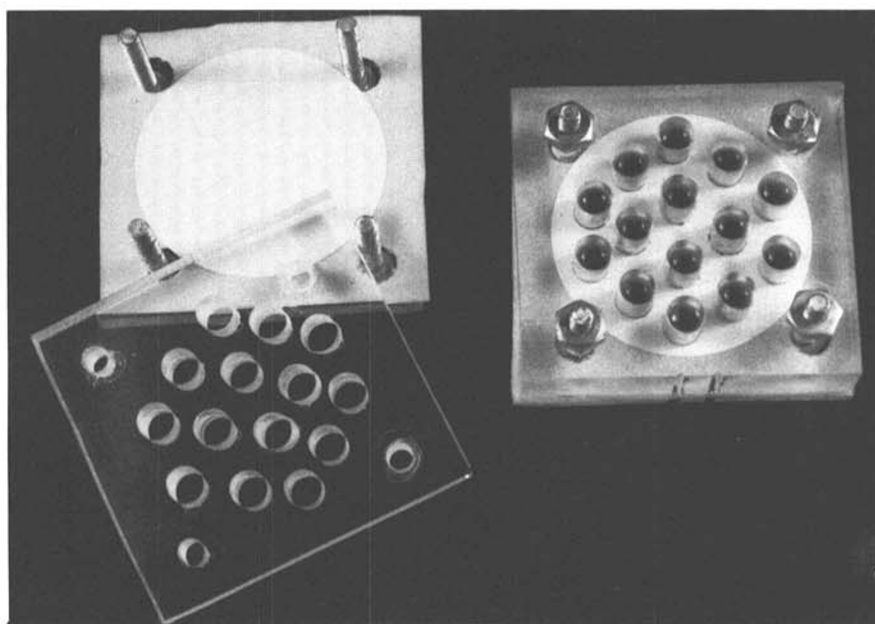

Fig. 1. (Left) A partially assembled and (right) an assembled template charged with sap extracts and additives. The nitrocellulose membrane over Whatman No. 1 filter paper is sandwiched between halves of the plastic template. Wells (5 mm diameter) were formed by drilling holes through the halves, permitting liquid to filter through the membrane and filter paper.

dilution, whereas the dilution end point was 1:16,000 for both viruses by DAS-ELISA. In dot-ELISA of both PVS and PVY, the membranes occasionally developed a faint pink color between dots that faded when the NCM were dried. The positive dots were easily distinguishable from negatives by both visual and reflectance measurements. Visual evaluation was almost as effective as reflectance measurements for differentiating positive and negative dots of dot-ELISA. Visual readings of color differences among wells using polystyrene cuvettes with DAS-ELISA were much less sensitive. Usually, color differences could not be distinguished visually for PVX reactions in wells where sap dilutions exceeded 1:500–1:1,000.

Dot ELISA vs. DAS-ELISA for detecting PVS and PVX in field-grown plants. Initially, we encountered a marked drop in sensitivity in dot-ELISA for detecting PVS and PVX in leaf sap of field-grown potato plants. Whereas a dilution end point of 1:128,000 was usually obtained in assays of PVX in leaf sap of potato plants grown in the greenhouse during the winter, this diminished to about 1:4,000 in sap of potato leaves of plants grown in the field. Corresponding losses of sensitivity occurred for dot-ELISA of PVS. The one conspicuous difference observed between assays of winter greenhouse-grown and field-grown plants was that plant sap would not completely pass through the NCM in assays of field-grown plants. To overcome this problem, several modifications were tested. Prefiltering sap with a Whatman No. 1 filter paper, an 8-μm Millipore NCM, or a fiberglass backup filter (Sierra Cascade Hi-Vol Aerosol Sampler, Sierra Instruments, Carmel Valley, CA) was ineffective. Substituting a 1.2-μm pore size Millipore NCM for a 0.45-μm NCM did not improve results. When potato sap was centrifuged at 10,000 *g* for 10 min., it was significantly clarified and more readily filtered through the NCM; however, dot-ELISA sensitivity increased only slightly. Adding 0.2% Triton X-100, 1 M NaCl, 12% sodium sulfite, or 0.2 M diethanolamine to PVX-infected sap also did not improve the assay. Incorporation of an enzyme mixture: pectinase (100 units per milliliter) plus cellulase (50 units per milliliter) plus beta glucuronidase (100 units per milliliter) (Sigma) in 0.1 M sodium acetate buffer, pH 5.0, resulted in complete passage of sap fluid through the NCM, but assay sensitivities were not improved.

Sap filtration and sensitivity both improved significantly when 0.01 M disodium ethylenediamine-tetraacetate (NaEDTA) (Fisher Scientific Co., Fair Lawn, NJ) was added to TBS-T80 plus 0.01 M NaDIECA for PVS and to TBS-T80 plus 10.0% Na_2SO_3 for PVX and mixed 1:1 with plant sap before deposition into template wells. In four experiments in which field-grown potato plants were assayed for PVX, and in three experiments for PVS using the addition of NaEDTA, the dilution end points for PVX and PVS were 1:128,000 and 1:16,000, respectively (Table 2). The comparative dilution end points for identical samples assayed with DAS-ELISA for PVX and PVS were 1:4,000 and 1:2,000, respectively. Addition of NaEDTA to potato sap was effective in partially restoring the sensitivity of dot-ELISA to the levels originally obtained in assays of plants grown in the greenhouse in winter and spring.

Table 1. Detection of potato viruses S, X, and Y (PVS, PVX, and PVY) in leaf sap of greenhouse-grown potato plants by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and dot-ELISA

Sap dilution[a]	DAS-ELISA[b] (A_{405}) $\bar{X}$	DAS-ELISA[b] (A_{405}) Range	Dot-ELISA Reflectance (log 1/R)[c] $\bar{X}$	Dot-ELISA Reflectance (log 1/R)[c] Range	Dot-ELISA Visual evaluation[d]
PVX					
1:100	1.27	0.66–2.52	0.56	0.17–9.63	+,+,+,+
1:500	0.58	0.32–1.08	···	···	···
1:1,000	0.33	0.16–0.63	···	···	···
1:2,000	0.15	0.05–0.37	···	···	+,+,+,+
1:4,000	0.03	0.00–0.11	0.35	0.07–0.38	+,+,+,+
1:16,000	<0.01	0.00–0.01	···	···	+,+,+,+
1:32,000	0.00	0.00–0.00	0.12	0.01–0.15	+,+,0,+
1:64,000	0.00	0.00–0.00	0.06	0.00–0.09	+,+,+,0
1:128,000	0.00	0.00–0.00	0.03	0.00–0.05	+,+,0,0
Healthy	0.12	0.04–0.20	0.06	0.04–0.07	0,0,0,0
PVS					
1:100	2.03	1.61–2.35	0.60	0.11–0.87	+,+,+,+
1:500	0.79	0.60–0.94	···	···	···
1:1,000	0.57	0.25–1.12	0.34	0.10–0.51	+,+,+,+
1:2,000	0.26	0.08–0.86	···	···	···
1:4,000	0.09	0.07–0.12	0.10	0.05–0.22	+,+,+,+
1:16,000	0.06	0.00–0.08	0.09	0.01–0.20	+,+,0,+
1:32,000	0.00	0.00–0.00	0.03	0.00–0.10	+,0,0,+
1:64,000	0.00	0.00–0.00	0.01	0.00–0.03	+,0,0,0
1:128,000	0.00	0.00–0.00	<0.01	0.00–0.01	+,0,0,0
Healthy	0.18	0.05–0.34	0.09	0.05–0.14	0,0,0,0
PVY					
1:100	1.33	0.33–1.69	0.28	0.12–0.33	+,+,+
1:500	0.32	0.09–0.58	···	···	···
1:1,000	0.17	0.05–0.36	0.14	0.07–0.21	+,+,+
1:2,000	0.07	0.01–0.16	···	···	···
1:4,000	0.04	0.00–0.08	···	···	+,+,+
1:16,000	0.03	0.00–0.05	0.06	0.00–0.14	+,+,0
1:32,000	0.00	0.00–0.00	0.03	0.00–0.05	+,0,0
1:64,000	0.00	0.00–0.00	0.01	0.00–0.03	0,0,0
1:128,000	0.00	0.00–0.00	0.00	0.00–0.00	0,0,0
Healthy	0.14	0.03–0.28	0.12	0.09–0.14	0,0,0

[a] Dilutions were parts sap from infected foliage:parts sap from healthy foliage (v/v).

[b] Mean absorbance of three wells for each dilution in each of four, four, and three experiments for PVX, PVS, and PVY, respectively. Each number is the mean ($\bar{X}$) of the infected minus the $\bar{X}$ of the healthy plus 2 SD of the $\bar{X}$ of the healthy. Negative values are indicated as 0.00.

[c] Mean reflectance of dots in each of four, four, and three experiments for PVX, PVS, and PVY, respectively. Each number is the $\bar{X}$ reflectance of the infected minus the $\bar{X}$ reflectance of the healthy plus 2 SD of the $\bar{X}$ of the healthy. Reflectance density = log 1/R.

[d] + = Positive, 0 = negative, and ··· = concentration not assayed.

DISCUSSION

Dot-ELISA resulted in improvements in serological sensitivity for PVS, PVX, and PVY over that obtained by DAS-ELISA in polystyrene cuvettes. The dilution end point detection for PVX was increased at least eightfold over that obtained by DAS-ELISA in polystyrene cuvettes. Several factors may account for

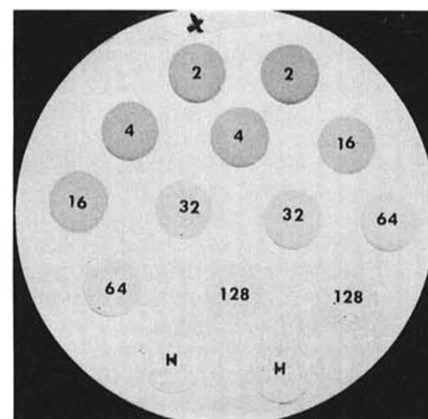

Fig. 2. A nitrocellulose membrane from dot-ELISA of potato virus X (PVX) from greenhouse-grown plants. The numbers are reciprocal dilutions ($\times 10^{-3}$, v/v) of PVX-infected potato in healthy potato leaf sap.

the increased sensitivity of dot-ELISA. First, the binding of antibody to the NCM is more efficient than it is to polystyrene plastics. Palfree and Elliott (11) found that murine Ia, a cell membrane glycoprotein, bound poorly to plastic when detergents were used to solubilize the cell membrane proteins, but 90% of the glycoprotein was bound to an NCM when a 0.01% concentration of detergent (Tween 80) was used. Desorption or "leakage" of antibody or antigen-antibody complexes from polystyrene plastics can further reduce the sensitivity of conventional ELISA. Salonen and Vaheri (13) found that only 20.9% of added human IgG was adsorbed to a polystyrene plastic surface if between 50 and 500 ng/ml of antibody was added. Of that adsorbed, only 50–70% remained attached by the end of the immunoassay.

Another factor that enhances detection of positive reactions in dot-ELISA is that the hydrolysis of substrates produces a bright stain that contrasts sharply against the light-colored NCM. Visual detection of the dots is highly sensitive, and unless quantitative measurements of reflectance among dots of different intensities are needed, photometric measurements appear unnecessary for high sensitivity.

Dot-ELISA is easier to perform than DAS-ELISA. Although 14- and 28-hole templates were used in most of these experiments, we have also satisfactorily used a 150-hole template. It should be possible to use templates of different sizes with different well numbers and configurations. The templates used in these experiments were inexpensive to prepare. Each NCM was coated and washed as a unit in a petri dish, and enzyme conjugates and substrates were applied in the same way. In assays in which a 150-hole template was used, only one-half as much (about 15 ml) enzyme conjugate was necessary, whereas 30 ml is required for the equivalent number of DAS-ELISA assays in polystytrene cuvettes. Only antigens must be added individually to each well of the template. The templates were cleaned; reused NCM are more economical than polystyrene plates or cuvettes. Visual evaluations are adequate for some applications. Therefore, this technique will reduce ELISA costs significantly. Filtration devices that may be effective for this technique are also available commercially (Millititer, Millipore Corp.; and Bio-Dot, Bio-Rad Corp.).

The reason for the large drops in sensitivity of dot-ELISA in assays for PVS and PVX from field-grown plants is not completely understood. It is likely that high temperatures and drought in the field in 1983 diminished virus titers in potato plants. The effect of NaEDTA in at least partially restoring sensitivity is not clear except that it chelates Mg^{++} and diminishes aggregation of organelles. It has also been shown to release plant viruses from cell organelles. Near the end of these experiments, we found that heating the plant sap:buffer-additive mixtures at 50 C for 10 min before depositing them on the template further improved sap filtration and increased color reactions (14).

Table 2. Detection of potato viruses S and X (PVS and PVX) in leaf sap of field-grown potato plants by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and dot-ELISA

Sap dilution[a]	DAS-ELISA[b] (A_{405})		Dot-ELISA Reflectance (log 1/R)[c]		Dot-ELISA Visual evaluation[d]
	$\bar{X}$	Range	$\bar{X}$	Range	
PVX					
1:100	1.09	0.58–2.24	...	...	...
1:500	0.49	0.38–1.19	...	...	...
1:1,000	0.28	0.12–0.77	...	...	...
1:2,000	0.13	0.05–0.26	0.34	0.23–0.40	+,+,+,+
1:4,000	0.07	0.04–0.11	0.26	0.19–0.33	+,+,+,+
1:16,000	0.00	0.00–0.00	0.17	0.14–0.19	+,+,+,+
1:32,000	0.00	0.00–0.00	0.12	0.07–0.16	+,+,+,+
1:64,000	0.00	0.00–0.00	0.10	0.05–0.13	+,+,+,+
1:128,000	0.00	0.00–0.00	0.09	0.07–0.12	+,0,+,+
Healthy	0.11	0.04–0.20	0.03	0.02–0.04	0,0,0,0
PVS					
1:100	0.84	0.63–1.18	...	...	...
1:500	0.27	0.07–0.43	...	...	...
1:1,000	0.12	0.04–0.19	0.16	0.06–0.21	+,+,+
1:2,000	0.04	0.00–0.11	0.09	0.05–0.13	+,+,+
1:4,000	0.00	0.00–0.00	0.07	0.02–0.09	+,+,+
1:16,000	0.00	0.00–0.00	0.01	0.00–0.03	0,0,0
1:32,000	0.00	0.00–0.00	0.00	0.00–0.00	0,0,0
1:64,000	0.00	0.00–0.00	0.00	0.00–0.00	0,0,0
1:128,000	...	...	...	...	...
Healthy	0.19	0.09–0.24	0.08	0.05–0.10	0,0,0

[a] Dilutions were parts sap from infected foliage:parts sap from healthy foliage (v/v). Equal volumes of 0.01 M NaEDTA + 10% Na_2SO_3 in TBS-T80 and 0.01 M NaEDTA + 0.01 M NaDIECA in TBS-T80 were added to each sap dilution for PVX and PVS, respectively, before it was deposited into template wells.

[b] Mean absorbance of four wells for each dilution in each of four and three experiments for PVX and for PVS, respectively. Each number is the mean ($\bar{X}$) of the infected minus the $\bar{X}$ of the healthy plus 2 SD of the $\bar{X}$ of the healthy. Negative values are indicated by 0.00.

[c] PVX dilutions of 1:2,000–1:128,000 and PVS dilutions of 1:1,000–1:64,000 were assayed in each of four and three experiments for PVX and PVS, respectively. Each number is the $\bar{X}$ reflectance of the healthy plus 2 SD of the $\bar{X}$ of the healthy. Reflectance density = log 1/R.

[d] + = Positive, 0 = negative, and ··· = concentration not assayed.

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