Detection of Potato Viruses X and Y in Sap Extracts by a Modified Indirect Enzyme-Linked Immunosorbent Assay on Nitrocellulose Membranes (NCM-ELISA)

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ABSTRACT

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Potato virus X (PVX) and potato virus Y (PVY) were detected in sap extracts from potato leaves by a modified indirect form of enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA). The procedure used monoclonal antibodies (MAs) originating from hybridoma clone C-9 for PVY and from clones 58, 59, and 67 for PVX. These MAs performed well in NCM-ELISA, reacting with similar virus and strain specificity in direct or indirect forms of ELISA. Sensitivity of NCM-ELISA was similar to that obtained with the direct double antibody sandwich form of ELISA when 10 and 200 µl of sap extracts, respectively, were used. Addition of 0.02 M disodium ethylenediaminetetraacetate in the extraction buffer increased sensitivity of NCM-ELISA. PVY was detected satisfactorily on NC membranes spotted with PVY-infected samples, sent airmail to Lima, Peru, from Guatemala, Brazil, and Uruguay, as well as on those kept locally at room temperature up to 20 wk before processing. The simplicity of this NCM-ELISA procedure allows its use in modestly equipped laboratories.

Breeding for resistance to potato virus X (PVX) and potato virus Y (PVY) in potato is one of the best methods for controlling these viruses. To improve the evaluation of virus-resistant germ plasm exposed in the field, the applicability of enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA) (5) was tested. This technique is essentially the same as dot-immunobinding assay (DIBA) (10). If NCM-ELISA could be applied successfully to detection of PVX and PVY, collaborators working in areas where well-equipped laboratories are not available could sample the test plants and mail spotted NCMs to a central laboratory for processing.

In initial tests, procedures were followed that had been described for assays similar to NCM-ELISA reported for the detection of PVY in purified preparations (4) or of other viruses (11,16). Partially purified polyclonal antibodies, which were satisfactory for the direct double antibody sandwich form of ELISA (DAS-ELISA) (6), produced nonspecific reactions in our NCM-ELISA.

In the experiments reported herein, the use of monoclonal antibodies (MAs) to avoid nonspecific reactions was investigated. A portion of this study was reported earlier (15).

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MATERIALS AND METHODS

Test plants and viruses. *Nicotiana glutinosa* L. and *N. occidentalis* Wheeler infected with PVX and PVY, respectively, and grown in the greenhouse for 3–4 wk were used as sources of these viruses. Infected potato cultivars grown in the field or greenhouse were also tested. The origin of the virus isolates was reported previously (3,7).

Antibodies. Polyclonal antibodies for PVX and PVY, produced in rabbits, were obtained from J. Nakashima (International Potato Center, Lima, Peru). Immunoglobulin G (IgG) and its alkaline phosphatase (Type VII-S, Sigma Chemical Co.) conjugate were prepared essentially as described by Clark and Adams (6), except that after dialysis, the IgG was not further purified.

Rat anti-PVX MAs originating from hybridoma clones 58, 59, and 67 were provided by L. Torrance (MAFF Harpenden Laboratory, England) and were supplied as partially purified IgG (17). Mouse anti-PVY MA originated from the Swiss hybridoma clone C-9 (8). The IgG and its alkaline phosphatase conjugate were purchased from Bioreba AG (Basel, Switzerland). Commercial goat antirabbit and goat antimouse IgG-alkaline phosphatase conjugates (Sigma) were also used.

Other materials. NCM pore size 0.45 µm, tris base, disodium ethylenediaminetetraacetate (EDTA), bovine serum albumin (BSA), *p*-nitro blue tetrazolium (NBT), *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), polyvinylpyrrolidone 40 (PVP), Tween 20, and nonfat dry milk were used.

Substrate was prepared in 0.1 M tris base buffer adjusted to pH 9.5 with 5 N HCl and 0.1 M NaCl, and 5 mM $MgCl_2$ was added. Color development solution was simplified from the method of Leary et al (14). The solution was prepared by dissolving 10 mg of NBT in 30 ml of substrate buffer protected from light. Immediately before use, 5 mg of BCIP dissolved in 100 µl of *N,N*-dimethylformamide was added in 10-µl drops to the NBT solution while the mixture was being shaken.

ELISA. Direct DAS-ELISA, as described by Clark and Adams (6), was used as the control to check the sensitivity of NCM-ELISA. Sap from leaf samples was extracted in phosphate-buffered saline plus 0.05% Tween 20 (pH 7.4) containing 1% ovalbumin and 2% PVP. Anti-PVY monoclonal and polyclonal IgG were used at 1 µg/ml, and their alkaline phosphatase conjugates were diluted 1:1,000. Anti-PVX polyclonal IgG was used at 1 µg/ml and its alkaline phosphatase conjugate was diluted 1:1,500. IgG of MAs 58, 59, and 67 was used at 2, 2, and 1.5 µg/ml, respectively. In DAS-ELISA for PVX with MA, microtiter plates were coated with MA and an anti-PVX polyclonal IgG-alkaline phosphatase conjugate was used to reveal trapped virus. Hydrolysis of *p*-nitrophenyl phosphate, 0.67 mg/ml, was measured colorimetrically (A_{405nm}).

General NCM-ELISA procedure. Samples of three or four potato leaflets or *Nicotiana* leaves were collected in 10× 15 cm plastic bags, and TBS (20 mM tris base, 500 mM NaCl, pH 7.5) was added to make a 1:10 (w/v) dilution. Sap extracts were prepared by rolling a test tube over the plastic bag. Extraction and all following steps were performed at room temperature, unless otherwise stated. Soaking, washing, and incubation were carried out in 9× 9 cm plastic plates. A grid 1× 1 cm was drawn on the 8× 8 cm NCM with a pencil. The NCM was handled with forceps or vinyl gloves and immersed 5 min in distilled water, then 5 min in TBS, with care taken not to entrap air. Two sheets of slightly larger No. 4

Whatman filter paper were soaked 10 min in TBS and placed on two dry Whatman filter papers. The NCM was placed on top and left to dry (1–2 min). Faster spotting and better appearing dots were obtained in this way. Antigen solutions (10 μl) were dotted onto each square and allowed to dry. The NCM was then transferred onto another dry filter paper for a further 2 hr of drying.

Table 1. Comparison of NCM-ELISA and DAS-ELISA using anti-PVY monoclonal antibody C-9 for the specific detection of PVY strains in *Nicotiana occidentalis* leaf sap extracts

Potyvirus isolate	Origin[a]	ELISA[b]	
		NCM[c]	DAS[d]
T (PVY^O)	Peru, La Molina	+	>2
SF (PVY^O)	Peru, La Molina	+	>2
255 (PVY^O)	Peru, La Molina	+	>2
218 (PVY^O)	Peru, Cañete	−	0.12
220 (PVY^O)	Peru, Cañete	+	>2
221 (PVY^O)	Peru, Cañete	−	0.17
224 (PVY^O)	Peru, Cañete	+	>2
H13 (PVY^O)	Peru, Cajamarca	+	>2
2 (PVY^O)	Chile	+	>2
171 (PVY^O)	Chile	+	>2
189 (PVY^O)	Chile	+	>2
52 (PVY^O)	Ecuador	+	>2
357 (PVY^O)	United States	−	0.12
CC5 (PVY^N)	Peru, Cuzco	+	>2
140 (PVY^N)	Peru, Cuzco	+	>2
201 (PVY^N)	Peru, Cuzco	+	>2
240 (PVY^N)	Peru, Cuzco	+	>2
198 (PVY^N)	Peru, Huanuco	+	>2
15.7 (PVY^N)	Peru, Huancayo	+	>2
129 (PVY^N)	Chile	+	>2
133 (PVY^N)	Chile	+	>2
8 (PVY^N)	Argentina	+	>2
36 (PVY^N)	Argentina	+	>2
38 (PVY^N)	Argentina	+	>2
48 (PVY^N)	Argentina	+	>2
2C (PVY^C)	Ecuador	+	>2
85N (PVY^C)	Ecuador	+	>2
Y^C-Arran (PVY^C)	Holland	−	0.13
PTV	Peru	−	0.14
PVA-1	Germany	−	0.09
PVA-5	Chile	−	0.09
PVA-327	Holland	−	0.08
PVA-328	Holland	−	0.12
PVV (PVY^C-GL)	Holland	−	0.12
PVV (PVY^C-AB)	Northern Ireland	−	0.11
PVV (UF)	Peru	−	0.19
TEV	United States	−	0.08
Healthy	...	−	0.09

[a] As reported by Baulcombe and Fernandez-Northcote (3) and Fernandez-Northcote and Gugerli (7).
[b] Sap dilutions (w/v) of 1:10 and 1:100 in the respective extraction buffers for NCM-ELISA and DAS-ELISA were tested, with similar results; only data for the 1:10 dilution are presented.
[c] Visual observation from two replicates; + = positive, − = negative reactions.
[d] Average A_{405nm} from two replicates after 30 min of hydrolysis.

Table 2. Comparison of NCM-ELISA and DAS-ELISA using anti-PVX monoclonal antibodies for the detection of PVX strains in *Nicotiana glutinosa* leaf sap extracts

Monoclonal antibody	ELISA[a]	PVX strain		
		cp	HB	GUA-2
58	NCM	10^{4b}	10^4	10^4
	DAS	5×10^4	5×10^4	5×10^4
59	NCM	10^3	10^3	10^4
	DAS	5×10^4	5×10^4	5×10^4
67	NCM	5×10^4	5×10^4	10^4
	DAS	5×10^4	5×10^4	5×10^2

[a] In DAS-ELISA, rat anti-PVX monoclonal antibodies were used for coating microtiter plates. Rabbit anti-PVX polyclonal antibody-alkaline phosphatase conjugates were used to reveal trapped virus. Range of A_{405nm} in healthy sap dilutions, from 1:10 up to $1:5 \times 10^4$, was 0.06–0.14 for all three MAs. Range of A_{405nm} at the dilution end points was 0.23–0.37.
[b] Reciprocal of dilution end point from two replicates at which a reaction was observed after 30 min of incubation in the color development solution for NCM-ELISA and of hydrolysis for DAS-ELISA when A_{405nm} readings were taken.

The NCM was put in 15 ml of the blocking buffer 3% BSA in TBS (5% dry nonfat milk was also satisfactory) for 1 hr, then transferred into TBS for a brief wash. Then, 250 μl per spot of monoclonal or polyclonal IgG (at the same concentration as for DAS-ELISA) in TBS plus 2% PVP and 0.2% BSA was added and the membrane incubated overnight in a sealed plastic plate placed on a gyratory shaker at 50 rpm. The NCM was rinsed very briefly in TTBS (TBS with 0.05% Tween 20), then washed three times (10 min each) with TTBS, 3 ml/cm^2 of membrane. Membranes were incubated 1 hr with goat antirabbit (for polyclonal antibodies) or goat antimouse (for monoclonal antibodies) IgG-alkaline phosphatase conjugates diluted 1:1,000 in the antibody buffer, rinsed briefly with TTBS, and washed as described above. Color development solution (250 μl per spot) was applied to the membranes and incubated 30 min to 1 hr.

Membranes were washed three times with distilled water, placed on dry filter paper, and dried. Positive reactions appeared as bluish purple spots, and negative reactions were either greenish or colorless.

RESULTS

Sample extraction and preparation. Initially, samples were collected and ground in mortars or extracted in the plastic bags in which they were collected. These extraction methods resulted in no differences in sensitivity and clarity.

Some greening and browning of spots obscured positive reactions and decreased the contrast between positive and negative reactions and the sensitivity of the NCM-ELISA. To overcome greening and browning, several sap treatments were compared. Sap was: 1) centrifuged 10 min at 12,000 *g*, 2) allowed to sit for 2 hr at 4 C, or 3) frozen at −4 C and allowed to sit for 24 and 48 hr. In the subtreatments, sap was: a) undiluted or mixed with 0.5 volume of b) TBS, c) TBS with 0.75% Na_2SO_3, or d) TBS with 0.01 M EDTA. Treatment 1 was the best, but treatment 2 and treatment 3d gave satisfactory results. Further improvement was obtained by extracting samples in TBS with 0.02 M EDTA and letting them sit for 30 min to 2 hr at 4 C before they were spotted on the NCM. Positive reactions obscured by greening could be revealed by rinsing the NCM with distilled water and by bleaching with 2% sodium hypochlorite for 10 min immediately after color development.

Comparison of NCM-ELISA with DAS-ELISA for detection of a broad spectrum of PVY isolates and of distinct PVX strains. Collected samples were ground, and sap aliquots were immediately diluted in the extraction buffer for NCM or DAS-ELISA. Ten, 12, and two isolates from the PVY^O, PVY^N, and

PVY^C groups of strains, respectively, mostly from the Andean region, were clearly detected by both NCM and DAS-ELISA (Table 1); anti-PVY MA C-9 was used in both assays. Isolate PVY^C-Arran from Holland and three PVY^O isolates (218 and 221 collected in Canete, Peru, by the second author and 357 obtained from G. V. Gooding, North Carolina State University) were negative by both NCM and DAS-ELISA. Potato virus A (PVA), potato virus V (PVV: PVY^C-GL, PVY^C-AB, and UF isolates), Peru tomato virus (PTV), and tobacco etch virus (TEV) were also negative in both tests. Isolates that gave negative reactions in our tests with MA reacted positively with the virus-specific polyclonal antibodies in DAS-ELISA (*data not shown*).

PVX isolates cp, HB, and GUA-2 were detected in NCM-ELISA (Fig. 1) by MA 58. MA 59 reacted strongly with GUA-2, a non-Andean strain, and weakly with cp and HB, both Andean strains. MA 67 reacted strongly only with isolates cp and HB. In the latter case, sensitivity was similar in both NCM and DAS-ELISA (Table 2). Polyclonal antibodies for PVX were also satisfactory for detecting all three PVX strains in NCM-ELISA (*data not shown*).

Sensitivity of NCM-ELISA for detecting PVX and PVY. The sensitivities of NCM-ELISA and DAS-ELISA were compared, using *N. glutinosa* grown in the greenhouse or potato cultivars with different levels of resistance to PVX and PVY inoculated with these viruses and grown in the greenhouse or field. Foliage samples were ground and sap dilutions were prepared immediately in the respective extraction buffers for NCM or DAS-ELISA. Results from at least three experiments for each virus indicated similar sensitivity for both tests. Data from one of the experiments for each virus are shown in Tables 2 and 3.

Effect of storage and mailing before processing of spotted NCM. NCMs were spotted with PVY-infected potato sap samples and stored, one set for 1, 2, and 7 days and two other sets for 1, 2, 4, 8, and 20 wk at room temperature to determine the sensitivity of the NCM-ELISA. PVY was detected in membranes stored up to 20 wk.

Two membranes were spotted with PVY-infected samples and sent airmail to Peru from Guatemala, Brazil, and Uruguay; 13, 15, and 19 days, respectively, elapsed from spotting to mailing and processing. All PVY-infected samples were detected.

To test whether infectious virus could be recovered from dried NCMs, samples were spotted and the NCMs were dried and stored for 2 hr to 8 wk. The NCMs were then soaked in blocking buffer for 1 hr; the blocking buffer, undiluted or diluted 1:10 or 1:100 with distilled water, was tested for infectivity by inoculating *N. occidentalis* plants. No infectivity could be recovered from NCMs containing samples from PVY-infected plants. However, infectious PVX was recovered from NCMs that had been stored for 4 wk.

DISCUSSION

MAs were used successfully to detect PVX and PVY in sap samples by a modified indirect NCM-ELISA (9), avoiding one of the main problems of the NCM-ELISA, namely, nonspecific reactions (12) usually obtained with polyclonal antibodies whose quality has not been improved by purification. To this aim, two other alternatives involving antisera processing have been recently reported for assays similar to NCM-ELISA. These are the cross-adsorption of antisera with plant constituents (4,13,16) and the use of $F(ab')_2$ fragments (1). With the NCM-ELISA procedure herein described, however, some

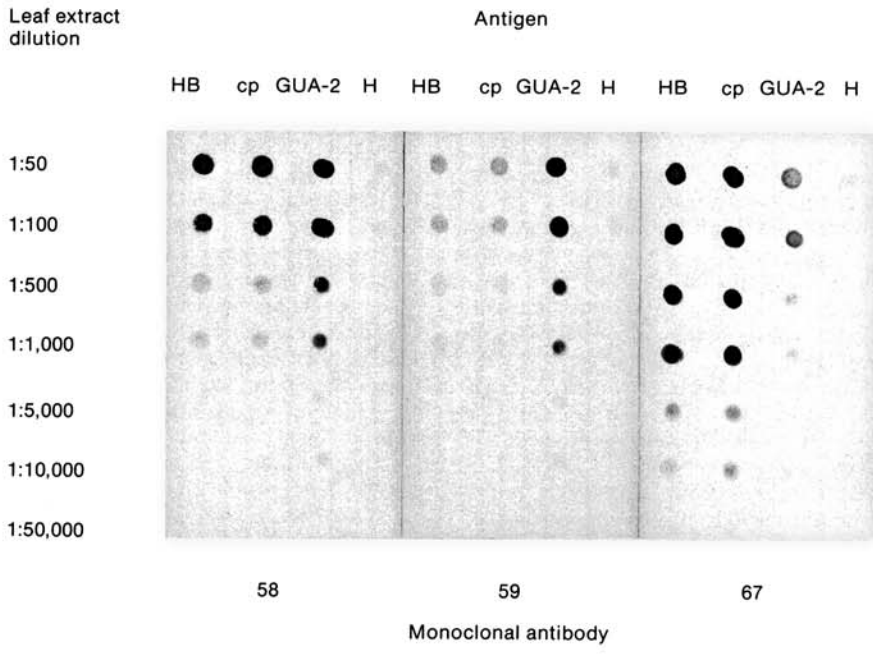

Fig. 1. ELISA on nitrocellulose membranes (NCM-ELISA) to detect potato virus X (PVX) strains in *Nicotiana glutinosa* leaf extracts. Antigen samples are the HB, cp, and GUA-2 strains of PVX; H shows extracts from healthy *N. glutinosa*. Monoclonal antibody 58 (MA 58) reacts strongly with all three strains; MA 59 reacts strongly with GUA-2 (a non-Andean strain) and weakly with HB and cp (both Andean strains); and MA 67 reacts strongly with HB and cp and weakly with GUA-2. Reaction is purple or light purple; no color reactions were observed under H, although spots remained light green at lower dilutions.

Table 3. Comparison of NCM-ELISA and DAS-ELISA using anti-PVY monoclonal antibody C-9 for the detection of PVY in potato leaves 14 wk after inoculation of greenhouse-grown plants

		Sap dilution[a]					
		1:10		$1:10^3$		$1:10^4$	
Potato clone	Plant no.	NCM[b]	DAS[c]	NCM	DAS	NCM	DAS
700367	1	++	1.46	+	0.09	+	0.00
	2	++	1.45	+	0.11	+	0.01
	3	++	1.51	+	0.06	−	0.02
382433.8	1	++	0.92	+	0.05	−	0.00
	2	+++	1.29	+	0.10	−	0.02
	3	+++	1.26	+	0.17	−	0.00
278072.1	1	++	0.38	+	0.06	−	0.02
	2	++	1.03	+	0.17	+	0.08
	3	++	1.14	+	0.19	−	0.07
703262	1	++++	1.41	+++	0.34	+	0.21
	2	++++	0.97	+++	0.42	+	0.32
	3	++++	1.39	+++	0.61	+	0.54

[a] Leaf sap dilution (w/v) in the respective extraction buffers for NCM-ELISA or DAS-ELISA.
[b] Visual observation from two replicates for each plant; + to ++++ = increasing intensities of positive reactions, − = negative reactions.
[c] Average A_{405nm} from two replicates for each plant after 30 min of hydrolysis, minus average from two replicates of uninoculated control. Range of A_{405nm} in sap dilutions from 1:10 up to $1:10^4$ for uninoculated controls was 0.04–0.06. Conjugate was anti-PVY monclonal antibody C9-alkaline phosphatase.

unprocessed polyclonal antibodies (probably of high quality) may be used successfully, as was the polyclonal for PVX in this work.

One of the main limitations on the use of MAs is that they might not function well in every type of immunoassay (19). The MA for PVX and PVY used here reacted with the same virus specificity and with either narrow or broad strain specificity as reported before in direct or indirect DAS-ELISA (7,8,17,18). In this work, however, in addition to PVY^{C}-Arran, three PVY^{O} isolates were identified that did not possess the epitope recognized by MA from hybridoma clone C-9 (7). Previous evidence (7,8) indicates that this kind of isolate is not important at present. The specificity of MA 67 for the Andean PVX isolates cp and HB was useful but partial, since in our studies it reacted weakly with a non-Andean PVX isolate. Torrance and Pead (18) reported that MA 67 reacted weakly with only five of 33 PVX isolates in indirect DAS-ELISA when tissue culture supernatant fluids were used at dilutions of less than 1:4. These five isolates, however, did not react in direct DAS-ELISA, even when substrate hydrolysis was allowed to continue for more than 16 hr.

Using polyclonal antiserum cross-adsorbed with healthy plant proteins and extending substrate incubation to 15 hr, Berger et al (4) reported the detection of 0.5 pg of PVY in purified preparations, by a test (DIBA) similar to our modified indirect NCM-ELISA. Banttari and Goodwin (2) reported that DOT-ELISA (a direct NCM-ELISA variant using IgG not passed through DE cellulose) was more sensitive than direct DAS-ELISA for the detection of PVX, PVY, and potato virus S (PVS) in leaf sap of greenhouse-grown potato plants (especially in the case of PVX) and for PVX and PVS in leaf sap of field-grown potato plants. Banttari and Goodwin (2) spotted 400 μl of sap plus additive using plastic templates to confine plant sap on wells on the NCM. Our NCM-ELISA procedure of spotting with 10 μl of plant sap diluted 1:10 in buffer directly on the NCM was usually as sensitive as DAS-ELISA in which 200 μl of plant sap diluted 1:10 in buffer was used. However, if plant sap was centrifuged 10 min at 12,000 *g* after extraction in TBS with 0.01 M EDTA, greater sensitivity was obtained with NCM-ELISA. The beneficial effects of EDTA were previously reported for the detection of PVX and PVS in potato leaf sap extracts by Banttari and Goodwin (2).

Koenig and Burgermeister (12) have mentioned some advantages of the NCM-ELISA. Our modified indirect NCM-ELISA is a simple testing procedure particularly suited for use in modestly equipped laboratories. Also, antigen-spotted NCMs are suitable for mailing to a central laboratory for processing. Because the samples mailed do not involve growing plants, any risk of disseminating infectious virus is avoided or could be eliminated, in the case of a stable virus like PVX, by sterilizing materials involved in processing the membrane. Mailing should facilitate and enhance international reciprocal collaboration in different areas of PVX and PVY virus research, such as detection of specific epitopes, variability, and epidemiology.

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