

# Optimization of a Chemiluminescent Dot-Blot Immunoassay for Detection of Potato Viruses

Ana C. Fulladolsa Palma · Rajitha Kota ·  
Amy O. Charkowski

Published online: 29 March 2013  
© Potato Association of America 2013

**Abstract** Vegetative propagation of potato leads to virus accumulation, resulting in significant yield losses and reduced quality. Virus identification is critical for developing disease management strategies and measuring seed lot health. The most widely used method of virus diagnosis in seed potatoes is a post-harvest test, for which the enzyme-linked immunosorbent assay (ELISA) is often used. ELISA was previously modified by substituting microtiter plates with membranes to develop a more flexible and inexpensive assay. We optimized a dot-blot immunoassay with viral proteins bound to a polyvinylidene fluoride (PVDF) membrane and detection of the proteins with alkaline phosphatase-labeled antibodies and a chemiluminescence reagent. The assay was tested for detection of viruses of seven genera. We have also altered the assay by spotting an antibody array onto a PVDF membrane and tested it for its potential uses as a diagnostic tool for *Potato virus Y*, *Hosta virus X*, and *Potato leafroll virus*.

**Resumen** La propagación vegetativa de la papa conduce a la acumulación de virus, lo que resulta en pérdidas significativas de rendimiento y reducción en la calidad. La identificación de virus es crítica para desarrollar estrategias de manejo de enfermedades y medir la sanidad de un lote de semilla. El método más ampliamente usado para el diagnóstico de virus en papa-semilla es una prueba de postcosecha, para la cual frecuentemente se utiliza el ensayo de enzimas conjugadas (ELISA). La ELISA fue modificada previamente mediante la sustitución de las placas de microtitulación por membranas para desarrollar un ensayo más flexible y barato. Nosotros optimizamos un inmunoensayo de gota con proteínas virales ligadas a una membrana de fluor-polivinilideno (PVDF) y detección de las proteínas con anticuerpos etiquetados con

fosfatasa alcalina y un reactivo quimioluminiscente. El ensayo se probó para la detección de virus de siete géneros. También alteramos el ensayo mediante la localización de una colección de anticuerpos en una membrana PVDF y se probó para sus usos potenciales como una herramienta de diagnóstico para el virus Y de la papa, el virus X de *Hosta* (Agavaceae), y el virus del enrollamiento de la hoja de papa.

**Keywords** Potato virus · Dot-blot immunoassay · ELISA · Virus detection

## Introduction

Viruses are among the most common plant pathogens and because viruses may cause mild or transient foliar symptoms that can mimic nutrient deficiencies, they may also be the most overlooked pathogens in vegetable production. Potato is the most important vegetable crop worldwide and over 30 different viruses are known to infect potato (Salazar 1996; Stevenson et al. 2001). These viruses vary in phylogeny, symptomatology, vector species, and whether or not they are phloem limited. Fortunately, only a handful of potato viruses have major impacts on potato production and among these, *Potato virus Y* (PVY) is currently the most important potato virus in North America (Gray et al. 2010).

PVY is the type member of the *Potyviridae* family (Shukla et al. 1998) and is composed of a filamentous, flexuous particle and a ss(+)RNA genome of approximately 9.7 kb (Scholthof et al. 2011). More than 50 species of aphids can transmit PVY in a non-persistent manner (Radcliffe and Ragsdale 2002). PVY has a wide natural host range and can infect plants in 14 genera of Solanaceae (Kerlan 2006). Infected plants show symptoms that range from mild to severe mosaic, leaf drop, leaf crinkle, leaf chlorosis, leaf necrosis, cracking and necrotic rings on tubers (Gray et al. 2010).

A. C. Fulladolsa Palma · R. Kota · A. O. Charkowski (✉)  
Department of Plant Pathology, University of Wisconsin-Madison,  
1630 Linden Dr.,  
Madison, WI 53706, USA  
e-mail: acharkowski@wisc.edu

Most commercial potato varieties are not resistant to PVY. This virus is controlled through production of certified seed potatoes, which are inspected to help maintain a seed supply with a low incidence of PVY and other viruses. These viruses are maintained below threshold levels by producing potatoes in a limited generation system that begins with disease-free tissue culture plantlets and by regular inspections of the crop during and after harvest. Routine virus indexing, usually by enzyme-linked immunosorbent assays (ELISA), is an important part of maintaining low levels of PVY in the North American seed potato crop.

ELISA (Engvall and Perlmann 1971; Van Weemen and Schuur 1971; Clark and Adams 1977) is a robust and inexpensive technology that is easily learned and thus well-suited to vegetable production. Several variations on ELISA have been developed (for example, see Bantari and Goodwin 1985; Lizarraga and Fernandez-Northcote 1989; Lin et al. 1990; Makkouk et al. 1993; Hsu et al. 1995), but few have become widespread other than the dot-blot immunoassay (DBIA), which is used, for example, for routine detection of *Citrus tristeza virus* (Cambra et al. 2000). A direct tissue blotting assay has been shown to be a useful tool for detection of different potato viruses, in some cases superior to ELISA, but adoption of this diagnostic tool for routine testing of a large number of samples has not occurred (Samson et al. 1993). Here we describe an adaptation of the DBIA that relies on polyvinylidene fluoride (PVDF) membrane and chemiluminescence detection for plant viruses. We compare the efficacy and cost of the assay to ELISA for detection of PVY, the most limiting virus in seed potato production. We also show that this assay works with several other viruses that infect potato and other crops, including *Potato virus A* (PVA), *Clover yellow vein virus* (CYVV), *Potato virus X* (PVX), *Hosta virus X* (HVX), *Potato virus S* (PVS), *Potato leafroll virus* (PLRV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), and *Tobacco ringspot virus* (TRSV).

## Materials and Methods

**Plant Samples** Greenhouse-grown potato plants infected with PVY, PVA, and PLRV, and plants that remained healthy were used for the assays. Leaves infected with PVX and PVS were obtained from samples collected from a varietal field trial at the U.S. Potato Genebank (Sturgeon Bay, WI). Plants infected with TMV, CMV, CYVV, TRSV, and HVX, were obtained from the UW-Madison Plant Disease Diagnostic Clinic.

**Dot-blot Immunoassay** Sap was extracted from leaf tissue with a sap extractor (Bantari 1980), and collected in 1.7  $\mu$ L microcentrifuge tubes. For each sample, 100  $\mu$ L of sap were loaded into a separate well on a 96-well polystyrene microtiter

plate. Sap was then spotted onto polyvinylidene fluoride (PVDF) Immobilon-FL Transfer Membranes (EMD Millipore Corporation, Billerica, MA) with a pin replicator (V&P Scientific, Inc., San Diego, CA) when more than 15 samples were being tested. A pin spotter (custom made to resemble an individual pin on the pin replicator) or a disposable wooden applicator with a flat tip was used to spot samples when less than 15 samples were being tested. Samples were always spotted in duplicate or triplicate so that the sample signal could be distinguished from background signal. After air-drying, membranes were rinsed with a solution of Tris-buffered saline (TBS; 1.25 mM Tris base, 7.5 mM NaCl, 0.1 mM KCl, pH 7.4) containing 0.05 % Tween-20.

The membranes were blocked with TBS+0.25 % Tween-20+5 % nonfat, dry milk (Carnation™, Produits Nestlé, S. A., Vevey, Switzerland) for at least 2 h or until the membranes appeared water-soaked, with agitation at 4 °C. The buffer volume added was three times the volume required to cover the membrane within the container used, typically 60 mL in a hybridization tube for a 96-sample membrane of 13×9 cm in size. After incubation, membranes were rinsed twice with TBS+0.05 % Tween-20.

Membranes were then incubated with a solution of TBS+0.05 % Tween-20+5 % nonfat, dry milk, a 1:2000 dilution of anti-PVY detection antibody 4C3 (Ellis et al. 1996), and a 1:2000 dilution of alkaline phosphatase enzyme conjugate (Agdia, Inc., Elkhart, IN), for at least 4 h, with agitation at 4 °C. After incubation, membranes were rinsed twice with TBS+0.05 % Tween-20.

Three 10-min washes with TBS+0.05 % Tween-20 were performed with agitation. Membranes were removed from the buffer solution and placed on plastic wrap. CDP Star chemiluminescence reagent (GE Healthcare Bio-Sciences Corp.), a 1,2-dioxetane substrate that emits light when activated by alkaline phosphatase, was added over the membrane at a volume sufficient to cover the surface, typically 2.5 mL for a 96-sample membrane, and the membrane was incubated for 5 min. Chemiluminescence was recorded immediately after incubation with CDP Star on a Molecular Imager® ChemiDoc™XRS System (Bio-Rad Laboratories, Inc., Hercules, CA) with an exposure time of 150 to 180 s.

**Test with Different Blocking Agents** Nonfat, dry milk, bovine serum albumin (BSA), and a recombinant *Pseudomonas syringae* pv. *syringae* HrpZ protein were tested as blocking agents. A set of 46 samples, a PVY-positive control and a PVY-negative control were used to spot two replicate membranes which were then incubated using BSA as a blocking agent, and two replicate membranes incubated with nonfat, dry milk. BSA was used at 3 % concentration in solution with TBS+0.25 % Tween-20 for blocking and at 1.5 % concentration with TBS+0.05 % Tween-20 for incubation with antibody solution. Nonfat, dry

milk was used at a 5 % concentration in solution with TBS+0.25 % Tween-20 for blocking and with TBS+0.05 % Tween-20 for incubation with antibodies. A second set of 46 samples, PVY-positive and PVY-negative controls were used to spot two replicate membranes, which were incubated with nonfat, dry milk as previously described, and two replicate membranes for incubation with the HrpZ protein. The protein was obtained by pelleting bacterial cells by centrifugation from 10 mL of liquid culture of *Escherichia coli* strain pSYH10 (He et al. 1993) in LB (Bertani 1952). The pellet was heated to boiling in 50 mL of TBS+0.25 % Tween-20 or 0.05 % Tween-20 and cooled before membrane blocking and incubation with antibody solution, respectively.

**Containers for Membrane Incubation** Containers for membrane incubation varied in size and material. Glass pyrex dishes of approximately 1.5 quart volume capacity and plastic sandwich containers were used for 96-sample membranes of 13×9 cm in size. Blocking buffer and antibody solution volume used was 50 mL. 20 mL of rinsing buffer were used. The trays were agitated on a Gyrotory® Shaker (Model G2, New Brunswick Scientific, Edison, NJ) at 4 °C. 96-sample membranes were also incubated in hybridization tubes (Techne FHB11, Bibby Scientific US, Burlington, NJ), with 60 mL of blocking buffer, 25 mL of antibody solution, and 20 mL of rinsing buffer. The tubes were rotated in a hybridizer (Techne HB-1D, Bibby Scientific US, Burlington, NJ) at 4 °C. For 15-sample membranes or smaller, 20 mm glass tubes or 25 mL polypropylene conical centrifuge tubes were used. 10 mL of blocking buffer, 8 mL of antibody solution, and 10 mL of rinsing buffer were used in this case. The tubes were rotated on a Rollordrum™ (New Brunswick Scientific, Edison, NJ) at 4 °C.

**Composite Sample Sensitivity Test** Sap from five PVY-positive plants was extracted and serially diluted seven times in 2-fold dilutions with sap from a single PVY-negative plant. The non-diluted and diluted PVY-positive samples, as well as the PVY-negative sample, were spotted onto two replicate membranes, which were then processed following the DBIA protocol described above. The experiment was repeated with a different set of three PVY-positive plants, for which sap was serially diluted 11 times in 2-fold dilutions with sap from a single PVY-negative plant.

**ELISA** Enzyme-linked immunosorbent assays were done using the compound direct ELISA PVY PathoScreen® Kit (Agdia, Inc., Elkhart, IN), and following the manufacturer's instructions. After 1 h of incubation with the p-nitrophenyl substrate, absorbance was measured at 405 nm on an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA). Positive-negative thresholds were defined as 3X the absorbance value of the negative control.

**Comparison of DBIA and ELISA** Tubers of potato cv. Marcy collected from the field were grown in the greenhouse. Of these plants, three sets, each comprised of 76, 78, and 79 individuals, were used to carry out an experiment to determine the level of agreement between DBIA and ELISA. Sap from leaves of each set of plants, a PVY-positive plant, and a PVY-negative plant, was extracted and 100 µL were loaded into a separate well on a 96-well polystyrene microtiter plate. Each sample was assigned an identification code and the sample layout on the plate was recorded. The DBIA was done for each sample set and controls on two replicate membranes, following the DBIA protocol described above. ELISA was also done for each set and controls. Cohen's kappa coefficient was used to compare the results for both assays and was calculated using R statistical software version 2.15.0 (R Development Core Team 2013).

**Antibody Array** Capture antibodies (Agdia, Inc., Elkhart, IN) were spotted onto a PVDF membrane using a pin spotter. Blocking was done by incubating the membranes with TBS+0.25 % Tween-20+5 % nonfat, dry milk for at least two hours or until the membranes appeared water-soaked, with agitation at 4 °C. After incubation, membranes were rinsed twice with TBS+0.05 % Tween-20.

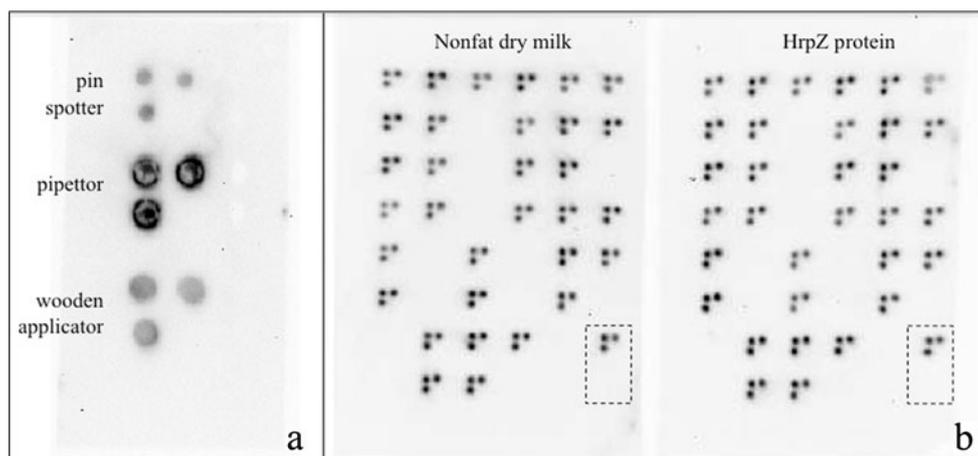
Membranes were then incubated with a solution of 1:1 sample sap to buffer solution (TBS+0.25 % Tween-20) for at least 4 h, with agitation at 4 °C. After incubation, membranes were rinsed twice with TBS+0.05 % Tween-20.

Membranes were incubated with a solution of TBS+0.05 % Tween-20+5 % nonfat, dry milk and a 1:2000 dilution of each detection and enzyme-linked antibodies (Agdia, Inc., Elkhart, IN), corresponding to the capture antibodies spotted onto the membrane, for at least 4 h, with agitation at 4 °C. After incubation, membranes were rinsed twice with TBS+0.05 % Tween-20.

Three 10-min washes with TBS+0.05 % Tween-20 and agitation were performed. Membranes were removed from the buffer solution and placed on plastic wrap. The membranes were incubated with CDP Star chemiluminescence reagent added over the membrane at a volume sufficient to cover the surface, typically 2.5 mL for a 96-sample membrane, for 5 min. Chemiluminescence was recorded immediately after incubation with CDP Star on an imaging station with an exposure time of 150 to 180 s.

## Results and Discussion

**Optimization of Sample Preparation** Different spotting tools were evaluated to identify an inexpensive and reproducible spotting method. Positive samples were most clearly detected when a flat-ended tool was used (Fig. 1a). A pin spotter was custom made for use in assays with 15 samples



**Fig. 1** Comparison of spotting tools and blocking agents. **a** PVY-positive signals obtained with different spotting tools: pin spotter, pipettor, and disposable wooden applicator. Tools with a flat end (pin spotter, wooden applicator) produced well-defined, solid spots that are easily interpreted. **b** No major differences were observed between

or fewer and was effective. Nevertheless, a disposable wooden applicator, which can be easily obtained, produced equally clear results. Spotting the plant sap with a pipettor produces spots with dark rings around them, which makes interpretation difficult.

We used a sap extractor to obtain the plant sap. No spotting buffer was required for this method; plant sap could be directly spotted onto membranes without further treatment. Plant sap samples could be stored at 4 °C for approximately 24 h, but assay sensitivity was greatly compromised when the samples were frozen.

**Optimization of Membrane Incubation** Membrane incubation was optimized for detection of PVY. Bovine serum albumin (BSA), nonfat, dry milk, and the recombinant *Pseudomonas syringae* pv. *syringae* protein HrpZ were evaluated as blocking agents. The blocking agents produced comparable results (Fig. 1b), with the exception that some batches of BSA resulted in unacceptably high background levels. We assayed HrpZ as a blocking agent after difficulty with reproducibility with BSA to determine if we could produce our own blocking agent. This protein is easily purified from cultured *E. coli* pSYH10 and is an effective blocking agent, but since nonfat, dry milk is an affordable and widely available product that did not vary in efficacy across batches, it was chosen for further assay optimization.

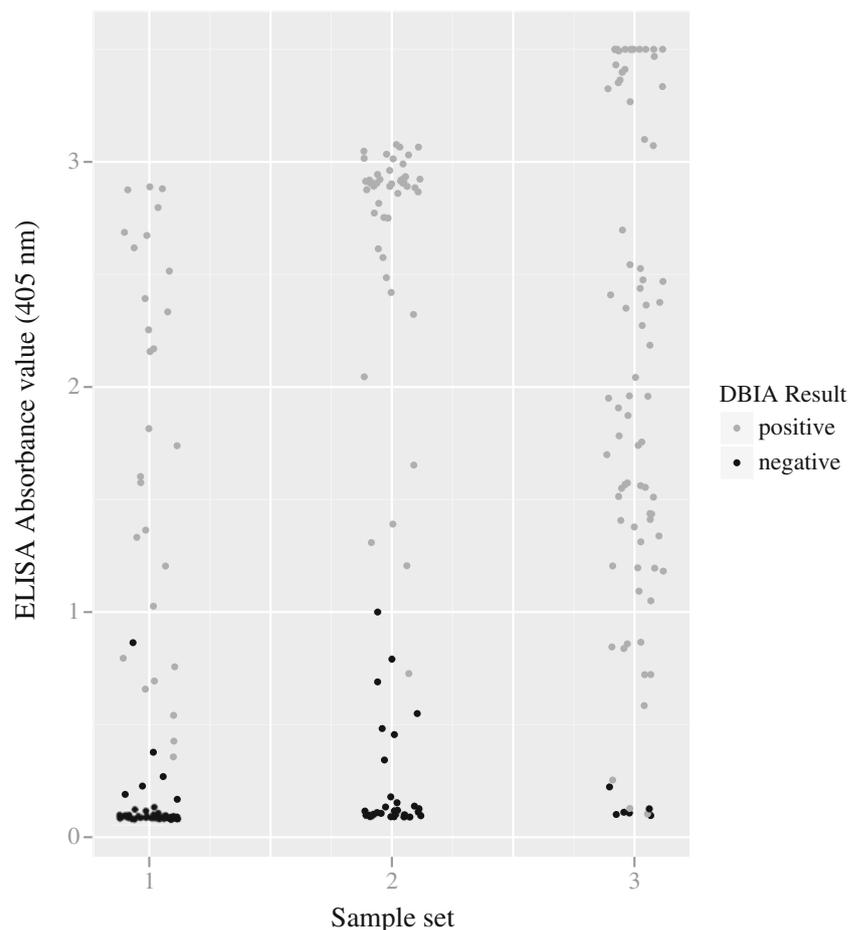
Incubation times with blocking buffer and with antibody solution were evaluated using small membranes with nine samples, a positive and a negative control. The experiment was performed twice with different samples. Incubations for all combinations of 1 h and 2 h in blocking buffer, and for 2 h and 4 h with antibody solution were carried out. The most reliable results were

obtained after incubation for 2 h with the blocking buffer and 4 h with antibody solution (results not shown). Lower incubation times result in background signal, possibly due to unspecific binding of the enzyme-linked antibodies to the membrane. Preliminary experiments using 1:1000 and 1:2000 dilutions of both detection and enzyme-linked antibodies showed that the higher dilution gave results as reliably as the lower one, so the 1:2000 dilution level was used for subsequent assays. Preliminary experiments also showed milk protein curdling at 20 °C and 37 °C; therefore we recommend incubation at 4 °C with agitation.

A variety of containers, including glass pyrex dishes, hybridization tubes, and smaller polypropylene and glass tubes, were used for the incubation steps depending on the size of the membranes, in order to reduce the volumes of blocking buffer and antibody solution required. In all cases, the surface of the membranes was fully exposed to the solution and there were no overlapping sections. The effectiveness of the assay was not compromised by the container used.

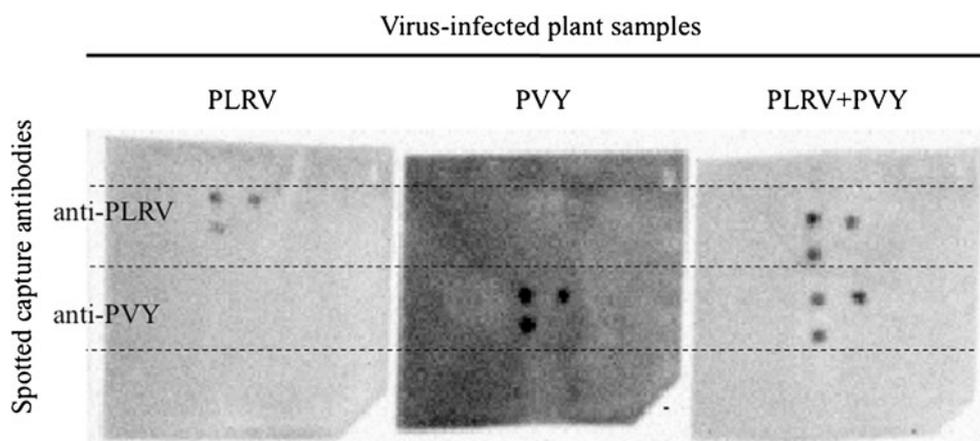
**DBIA Sensitivity when Samples are Compositated** Combining samples into a composite or bulk sample is a common practice when using conventional ELISA for virus detection. Two experiments were conducted to determine the number of samples that could be bulked without compromising the clarity of the results. In both experiments, spots corresponding to positive samples diluted up to 8-fold were detected, but spots were clearly visible for samples diluted by a factor of 4-fold or less (not shown). This is equivalent to bulking eight samples or less. At higher dilutions, the signal was weak and results were difficult to interpret.

**Fig. 2** ELISA absorbance values for three sets of potato leaf samples used for detection of PVY. *Black circles* and *gray circles* represent DBIA PVY-negative and PVY-positive results, respectively. Two of 76 samples in set 1 and seven of 78 samples in set 2 were PVY-negative on the DBIA and were above the ELISA positive-negative threshold absorbance values (405 nm) of 0.288 and 0.270, respectively for each set. Three of 79 samples in set 3 were PVY-positive on the DBIA and below the ELISA threshold absorbance value of 0.303



*DBIA vs. ELISA for Detecting PVY* ELISA has become the standard method used for post-harvest testing of leaf samples from seed lots. In order to compare the DBIA to the standard ELISA, three different sets of 76, 78, and 79

samples, and PVY-positive and negative controls were used to perform both assays. The percentage of samples that differed between assays was 2.6 %, 8.8 %, and 3.7 % for each data set respectively (Fig. 2). There was much more



**Fig. 3** Antibody array-based assay for detection of PLRV and PVY. PLRV and PVY capture antibodies were spotted onto three membranes. The membrane incubated with PLRV-infected plant sap showed a positive signal (represented by a three-spot pattern) only where PLRV capture antibodies were spotted (*left*). The membrane incubated with PVY-infected plant sap showed a positive signal only where PVY

capture antibodies were spotted (*center*). The membrane incubated with both PVY- and PLRV-infected plant sap showed a positive signal where PVY and PLRV capture antibodies were spotted (*right*). The assay was repeated with a second set of plant samples and similar results were obtained

agreement between ELISA and DBIA than expected by chance alone, as indicated by Cohen's kappa coefficients of 0.94, 0.81, and 0.78, respectively for each set.

The advantages of DBIA include that numerous assays can be completed with only a small sample volume, the method is simpler, and spotted membranes can be stored for a few days, and therefore can be mailed or carried from a field site to a laboratory. DBIA is also less expensive than ELISA. A commercial ELISA kit suitable for testing 480 samples currently costs \$400 or more, while DBIA can be completed in less time on the same number of samples for approximately \$75. However, unlike ELISA, DBIA is not quantitative.

The dot-blot immunoassay was successfully used to detect several genera of viruses in different hosts, including *Potyvirus* (PVY and PVA in potato, CYVV in clover), *Potexvirus* (PVX in potato, HVX in hosta), *Carlavirus* (PVS in potato), *Luteovirus* (PLRV in potato), *Cucumovirus* (CMV in pumpkin), *Tobamovirus* (TMV in *Nicotiana benthamiana*), and *Nepovirus* (TRSV in *N. benthamiana*). Signals from virus-infected samples were clearly visible for all of the samples tested, including those infected with PLRV, from which it is often difficult to detect a signal after ELISA due to low virus titer in the host plant.

**Antibody Array** Capture antibodies for PLRV and PVY were spotted onto three PVDF membranes. The first membrane was incubated with sap from a PLRV-infected plant sample, diluted in TBS+0.25 % Tween-20. The second membrane was incubated with sap from a PVY-infected sample, diluted in buffer, as indicated above. The third membrane was incubated with sap from both PLRV- and PVY-infected plant samples, diluted in buffer. All three membranes were incubated with an antibody solution that contained both PLRV and PVY detection and enzyme-linked antibodies. For the first membrane, a positive signal was detected only from the spots where PLRV capture antibody was spotted (Fig. 3). On the second membrane, a positive signal was detected only from the spots where PVY capture antibody was spotted. For the third membrane, a positive signal was detected from the spots where PLRV and PVY were spotted. This experiment was repeated a second time and the same results were produced. The antibody array was also successfully used for simultaneous detection of HVX and PVY. Therefore, this method also has potential as an inexpensive and simple method that can be used to screen a few plant samples for numerous viruses.

**Potential Uses of DBIA for Virus Indexing in Potato** The DBIA is an adequate and effective method for detecting different viruses in a small or large number of plant samples. Virus indexing is a crucial part of disease management in seed programs and this assay can potentially reduce the cost

of testing. We showed that the assay could be done with different materials (i.e. spotting tools, blocking agents, containers for membrane incubation), provided there is an adequate system for detection of chemiluminescence. The use of a 96-well format for arranging the sap samples, and furthermore, the use of a spotting tool to spot sap onto the membrane, offers a novel way to prepare the samples, allowing us to easily prepare replicate membranes that could be used for simultaneous detection of different viruses. The sap samples can be stored in an organized manner and can be used for confirmation assays if needed. In addition, chemiluminescent assays have been shown to produce results that can be interpreted more easily than those obtained by colorimetric assays, due to background caused by plant tissue staining in the latter (Makkouk et al. 1993). The use of chemiluminescence also allows us to easily record and store results.

All the experiments performed complied with the current regulations of the United States of America.

**Acknowledgements** We thank our funding sources, the USDA Specialty Crop Research Initiative Grant project number 2009-02768 and the Gloeckner Foundation. We also thank Kristi Severson, Brian Hudelson, Nicholas Keuler, and Kenneth Frost for their assistance with this work, and the anonymous reviewers who helped us improve our manuscript.

## References

- Banttari, E.E. 1980. Sap extraction device for sampling potato leaves and tubers. *American Potato Journal* 57: 345–348.
- Banttari, E.E., and P.H. Goodwin. 1985. Detection of potato viruses S, X, and Y by enzyme-linked immunosorbent assay on nitrocellulose membranes (Dot-ELISA). *Plant Disease* 69: 202–205.
- Bertani, G. 1952. Studies on Lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* 62: 293–300.
- Cambrá, M., M.T. Gorrís, C. Marroquín, M.P. Román, A. Olmos, M.C. Martínez, A. Hermoso de Mendoza, A. López, and L. Navarro. 2000. *Virus Research* 71(1–2): 85–95.
- Clark, M.F., and A.N. Adams. 1977. Characteristics of the microplate method enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475–483.
- Ellis, P., R. Stace-Smith, G. Bowler, and D.J. MacKenzie. 1996. Production of monoclonal antibodies for the detection and identification of strains of potato virus Y. *Canadian Journal of Plant Pathology* 18: 64–70.
- Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 8: 871–874.
- Gray, S., S. De Boer, J. Lorenzen, A. Karasev, J. Whitworth, P. Nolte, R. Singh, A. Boucher, and H. Xu. 2010. Potato virus Y: An evolving concern for potato crops in the United States and Canada. *Plant Disease* 94(12): 1384–1397.
- He, S.Y., H.C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. *syringae* Harpin<sub>PSs</sub>: A protein that is secreted via de Hrp pathway and elicits the hypersensitive response in plants. *Cell* 73: 1255–1266.

- Hsu, H.T., J.Y. Kim, and R.H. Lawson. 1995. Purification of lily symptomless carlavirus and detection of the virus in lilies. *Plant Disease* 79: 912–916.
- Kerlan, C. 2006. Potato virus Y. CMI/AAB Descriptions of plant viruses 414.
- Lin, N.S., Y.H. Hsu, and H.T. Hsu. 1990. Immunological detection of plant viruses and a mycoplasma-like organism by direct tissue blotting on nitrocellulose membranes. *Phytopathology* 80: 824–828.
- Lizarraga, C., and E.N. Fernandez-Northcote. 1989. Detection of potato viruses X and Y in sap extracts by a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA). *Plant Disease* 73: 11–14.
- Makkouk, K.M., H.T. Hsu, and S.G. Kumari. 1993. Detection of three plant viruses by dot-blot and tissue-blot immunoassays using chemiluminescence and chromogenic substrates. *Journal of Phytopathology* 139: 97–102.
- R Development Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. URL <http://www.R-project.org/>.
- Radcliffe, E.B., and D.W. Ragsdale. 2002. Aphid-transmitted potato viruses: The importance of understanding vector biology. *American Journal of Potato Research* 79: 353–386.
- Salazar, L.F. 1996. *Potato viruses and their control*. Lima: CIP. 214 p.
- Samson, R.G., T.C. Allen, and J.L. Whitworth. 1993. Evaluation of direct tissue blotting to detect potato viruses. *American Potato Journal* 70: 257–265.
- Scholthof, K.-B.G., S. Adkins, H. Czosnek, P. Palukaitis, E. Jacquot, T. Hohn, B. Hohn, K. Saunders, T. Candresse, P. Ahlquist, C. Hemenway, and G.D. Foster. 2011. Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology* 12(9): 938–954.
- Shukla, D.D., C.W. Ward, A.A. Brunt, and P.H. Berger. 1998. AAB Descriptions of plant viruses 366.
- Stevenson, W.R., R. Loria, G.D. Franc, and D.P. Weingartner. 2001. *Compendium of potato diseases*. St. Paul: APS Press. 106 p.
- Van Weemen, B.K., and A.H.W.M. Schuur. 1971. Immunoassay using antigen-enzyme conjugates. *FEBS Letters* 15: 232–236.