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DIAGNOSTICS OF THE PRESENCE OF VIRUSES IN THE POTATO AND SOIL

ABSTRACT

The paper presents a combined biological assay on indicator plants, a serological assay using ELISA and a molecular assay for detection of viruses that infect the potato. It also elaborates on the bioassay for PVY strain differentiation and for detection of TRV in tubers and soil samples. At IHAR-PIB Młochów Research Center, ELISA tests can be conducted for detection of PVY, PVA, PVM, PVS, PLRV, PVX, TRV and PMTV. Multiplex RT-PCR, real-time quantitative RT-PCR and sequencing methods for detection of PVY, TRV and PMTV are optimized based on a published protocol or developed in our laboratory. The primers for detection of PVY, TRV, PVM, PVS, PLRV, PVX, PMTV, PVA, AMV, CMV, PAMV, TBRV, and BMYV according to the published methods are listed. The maintenance and usage of 19 species of indicator plants are discussed.

Keywords: ELISA, bioassay, potato, RT-PCR, sequencing, virus

INTRODUCTION

There are more than 35 potato viruses known to infect the potato in the field (Valkonen, 2007). A virus infection may cause yield loss and decrease the tuber quality depending on the virus /strain, the potato genotype and environmental conditions. Table 1 shows the impact of 12 viruses on the potato crop. Among them, *Potato virus Y*, *Potato virus M*, *Potato virus S*, *Potato leafroll virus*, *Tobacco rattle virus* are frequently found in the tuber or soil samples collected from the Central or Northern part of Poland in field conditions. Other viruses are shown to infect the potato by artificial inoculation in greenhouse conditions, and some are only maintained in the collection at IHAR-PIB Młochów Research Center.

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Table 1

Yield loss and damage to potato tubers caused by viruses, based on Jeffries (1998) with slight modifications

Viruses	Abbreviations	Yield loss	Tuber necrosis or damage
Potato virus Y	PVY	10-80%	++
Potato virus M	PVM	15-45%,	b
Potato leafroll virus	PLRV	Up to 90%	++
Potato virus S	PVS	10-20%	b
Potato virus X	PVX	15-20%	+
Potato virus A	PVA	Up to 40%	b
Tobacco rattle virus	TRV	Loss of saleable yield	+++
Potato mop-top virus	PMTV	Yield loss by secondary infection	+++
Tomato black ring virus	TBRV	30-80%	+
Alfalfa mosaic virus	AMV	Little economic importance	+
Potato aucuba mosaic virus	PAMV	Little economic importance	+
Cucumber mosaic virus	CMV	Little economic importance	na

a : possible to induce tuber necrosis after grafting; b: not possible to induce tuber necrosis after grafting (Chrzanowska, 1993) ; na: not available, +++: most severe symptoms, ++: medium disease symptoms, +: weak symptoms.

The basic tests to detect viruses are: a bioassay on indicator plants and serology using an ELISA with polyclonal and monoclonal antibodies (Jeffries, 1998). Several viruses may be detected on a single indicator plant, whereas ELISA is virus- and strain-specific. Nucleic acid based methods such as RT-PCR and sequencing help to reveal the genomic feature of the virus or to identify a new one. Real-time quantitative RT-PCR is highly sensitive and can detect a virus at a very low concentration.

For identification of viruses that infect the potato, a combined biological assay on the indicator plants, a serological assay using ELISA and a molecular assay based on nucleic acid detection are required. The method for the bioassay is based on Muchalski and Chrzanowska (2001) with some modifications and based on the Descriptions of Plant Viruses (http://www.dpvweb.net/index.php) and Jeffries (1998).

MATERIALS AND REAGENTS

Biological assay

1) Soil mixed with peat in a proportion of 2:1 in trays.
2) Fertilizer PG mix (14-16-18) + Micro at a concentration of 0.5-0.8 kg/l m^3.
3) Pots (Ø=8cm)
4) Seeds of the indicator plants and test plants (see Table 2, e.g., tobacco

cv. Samsun, *Chenopodium amaranticolor, Ch. quinoa*)
5) Virus-free tubers of the indicator potato cultivars (e.g., cvs King Edward, Desiree, Pentland Ivory, Nicola)
6) Carborundum powder
7) Latex powder-free gloves "Protect clinic" (Semperit Technische Producte Gesellschaft)
8) Mortar and pestle

Serological assay (ELISA) (acc. Syller, 2001)

1) ELISA buffers
2) PBS (Phosphate-buffered saline) (pH 7,4)
   a) NaCl 8.0 g (POCH, cat. No 794121116)
   b) KH_2PO_4 0.2 g (POCH, cat. No 742020112)
   c) Na_2HPO_4 12 H_2O 2.9 g (POCH, cat. No 799280115)
   d) KCl 0.2g (POCH, cat. No 739740114)
   e) NaN_3 0.2g (CHEMPUR, cat. No 117927704)
   f) Adjust pH with either NaOH or HCl (POCH, cat. No 575283115) and make up with distilled water to 1 liter
3) PBS-Tween
   a) add 0.5-1ml Tween 20 per 1 l PBS (Sigma, cat. No P-1379-1L)
4) Coating buffer (pH 9,6)
   a) Na_2CO_3 1.59 g (POCH, cat. No 810570113)
   b) $NaHCO_3$ 2.93 g (POCH, cat. No 810530115)
   c) NaN_3 0.20 g (CHEMPUR, cat. No 117927704)
   d) Make up to 1 liter with distilled water
5) Extraction buffer (pH 7.4)
   a) PBS-Tween (as above)
   b) Polyvinylpyrrolidone (PVP) 2g per 1 l (Sigma, cat. No PVP 40T)
6) Conjugate buffer
   a) The same as the Extraction buffer (see above)
7) Substrate buffer (pH 9.8)
   a) Diethanolamine 97 ml (Sigma, cat. No D8885)
   b) Distilled water 600 ml
   c) NaN_3 0.5g (CHEMPUR, cat. No 117927704)
   d) Add Phosphatase substrate (Sigma, cat. No P4744) 0.75-0.1 mg per 1ml of substrate buffer.
   e) Antibodies:
      – PVY all strains - monoclonal cocktail antibody (Bioreba IgG, cat. No 112911; Bioreba Conjugate, cat. No 112921)
      – PVY^N – monoclonal antibody (Bioreba IgG, cat. No 112911; Bioreba Conjugate, cat. No 112722)
      – $PVY^{O/C}$ –monoclonal antibody (Bioreba IgG, cat. No 112911; Adgen Conjugate, cat. No 1052-04)
      – PVA (Hornik IgG, cat. No 9057568; Hornik Conjugate, cat. No 9057559)

- PVM (Adgen IgG, cat. No 1042-02; Adgen Conjugate, cat. No 1042-04)
- PVS (Bioreba IgG, cat. No 110311; Bioreba Conjugate, cat. No 110321)
- PLRV (Bioreba IgG, cat No 110611; Bioreba Conjugate, cat. No 110621)
- PVX (Bioreba IgG, cat. No 110411; Bioreba Conjugate, cat. No 110421)
- TRV (SASA, UK, TRV-PRN sets)
- PMTV (SASA, UK, PMTV-mAb sets)

Molecular assay

1) RNeasy Plant Mini kit (Qiagen, cat. No 74904)
2) Ethanol (96-100%) (ethanol 96% CZDA, PURA P.A., cat. No 396420113 (500 ML)
3) Superscript III one-step RT-PCR with Platinum Taq DNA polymerase (Invitrogen, cat. No 12574026)
4) Agarose (Sigma, cat. No A9539)
5) Ethidium bromide solution (Sigma, cat. No E1510-10ML)
6) TaqMan micro-RNA Reverse Transcription kit (Applied Biosystems, cat. No 4366597)
7) SYBR Select Master Mix (Applied Biosystems, cat. No 4472908)
8) Virus specific primers (see table 4, 5 and 6)
9) QIAquick purification kit (Qiagen, cat. No 28104)

EQUIPMENT

1) Insect-free greenhouse with natural light
2) Squeezing machine (Press for squeezing leaves, producer: SUDD-ELECTROMOTORENWERKE BRUCHSAL, TYP DFWZ-1B-1-2, NR 12408290)
3) 96-well micro-ELISA plates (MEDLAB 39-096f-OS)
4) ELISA reader (DYNATEX MRX II)
5) Pipette sets (Sartorius Poland Sp. z o.o.)
6) Deep freezer (- 80°C) (Frigor)
7) LightCycler 480 real-time PCR instrument (Roche Diagnostics)
8) LightCycler 480 Multiwell Plate 96, clear (Roche Diagnostics Polska Sp. z o.o., cat. No 05102413001)
9) Electrophoresis unit (B2 complete, Minigel System 12 x 14 cm, SYM-BIOS Sp. z o.o.)
10) Power supply (EC300XL2: Compact model, Max Output: 300V, Europe cord, SYMBIOS Sp. z o.o.)
11) GeneAmp PCR System 9700 (Applied Biosystems)
12) MiSeq sequencer (Illumina) (sequencing done by the DNA Sequencing Laboratory, IBB PAS, Poland)

PROCEDURE

The preparation of the indicator plants

1) Seeds of the indicator plants are sown into autoclaved soil mixed with peat in a proportion of 2:1 in trays. The soil mixture also contains Fertilizer PG mix (14-16-18) + Micro at a concentration of 0.5-0.8 kg × m^{-3}.
2) The rooted young seedlings are transplanted into pots (Ø=8cm). The plants at a suitable physiological stage are used in the assay.
3) If potato plants are used as the indicator, the tubers are first tested for the presence of viruses by ELISA in a growing-on test. Only the virus-free, healthy tubers are used for multiplication of the potato test plants.
4) All the plants are kept in an insect-free greenhouse under natural light conditions from May to August (day temp. 20-26°C and night temp. 14-16°C).
5) A list of the indicator plant species used for potato viruses diagnostics is shown in Table 2.

Table 2

The indicator plants used for detection of potato viruses at IHAR-PIB/Młochów. Based on Muchalski and Chrzanowska (2001) with modifications.

Species	Period [days] / [stage]			
	from sowing to transplanting	from transplanting to inoculation	Total	of greatest susceptibility to infection[d]
Capsicum annum L.	10-12	30-35	40-47	20
Chenopodium album L.	7-10	20-27	27-37	15
Ch. amaranticolor Coste et Reyn.	10-12	25-35	35-47	12
Ch. quinoa Willd.	10-12	20-28	30-40	12
Datura metel L.	8-12	18-23	26-35	15
D. stramonium L.	20-22[a]	25-30[a]	25-35[a]	20
Gomphrena globosa L.	10-14	20-24	30-38	Young leaves
Lycopersicon chilense Dun.	7-10	20-27	27-37	15
Nicotiana benthamiana[a]	20-25[a]	20-22[a]	40-48[a]	15 -20[a]
N. clevelandii Gray	16-24	18-23	34-47	15[a]
N. glutinosa L.	7-10	18-24	25-34	15[a]
N. debneyi Domin.	7-10	18-24	25-34	15
N. occidentalis P-1 Domin[b]	10-14	18-24	28-38	15
N. tabacum L. cv. Samsun	20-22[a]	18-20[a]	40-48[a]	15-20[a]
Phaseolus vulgaris cv. Red Kidney[a]	15-20[a]	15-20[a]	25-30[a]	10-15[a]
Physalis floridana Rydb.	10-15	20-28	30-43	20
Solanum demissum × Aquila (A-6)			35-40	Young leaves
S. lycopersicum[c]	15-20[a]	18-25	26-37	20
S. rostratum Dunal	7-10	18-25	25-35	18

[a] according to Michalak (not published); [b] according to Kryszczuk and Chrzanowska (2000); [c] cultivars: Najwcześniejszy, Newski, Rutgers; [d] when the plants have developed 2-4 fully expanded leaves

Identification of potato viruses in the indicator plants – a biological assay

1) A viral infection may cause characteristic disease symptoms in the indicator plants, which can be used for diagnosing a specific virus. In most cases, sap mechanical inoculation of the indicator plants can be used, except that PLRV must be transmitted by aphids (*Myzus persicae*) or by grafting.
2) Mechanical inoculation is performed using sap from systemically infected tobacco (cv. Samsun) leaf tissues 14 days post-inoculation (dpi) that were ground in 20 volumes of sterile water. Sometimes a phosphate buffer is used, e.g. 0.02 M (pH 7.4) or 0.06 M (pH 8.4).
3) The symptoms appear around 4-21 dpi. For example, based on our experiments, in *Chenopodium amaranticolor*, local necrotic lesions caused by PVY and TRV appear around 4-7 dpi. In *Ch. quinoa*, TRV causes local symptoms at around 4 dpi, and systemic ones around 10-14 dpi. In *Nicotiana tabacum*, veinal clearing (VCl) caused by PVY appears around 7-9 dpi, while veinal necrosis (VN) appears around 12-14 dpi. Systemic distortions, necrotic lesions and line patterns on the leaves caused by TRV in *N. tabacum* appear around 7-21 dpi depending on isolates.

The reactions of the indicator plants to infection by potato viruses are described and are shown in Table 3. The symptoms induced by viruses in potato are described separately.

Table 3

Indicator plants for detection of potato viruses (based on de Box 1987, Jeffries 1998, Verhoeven and Roenhorst 2003)

Species	Viruses					
	PVY	PLRV	PVM	PVS	PVX	PVA
Capsicum annum	S				L	
Chenopodium album	L		L	L	L	
Chenopodium quinoa	L		L	L	L	
Chenopodium amaranticolor	L		L	L	L	
Datura metel	L		L/S		L/S	S
Datura stramonium		S*		S*	L/S*	
Gomphrena globosa			L	L	L*	
Lycopersicon chilense			S*	S	S	
Nicotiana benthamiana	S				L/S	S
Nicotiana clevelandii	S				S	S
Nicotiana debneyi	S		L/S	S*	S	S
Nicotiana glutinosa	S				S	S
Nicotiana tabacum cv Samsun	S*				L/S*	S*
Nicotiana occidentalis P-1	L?S			LS	LS	
Phaseolus vulgaris			L			
Physalis floridana	L/S	S*				S
Solanum rostratum			L/S*			
Solanum lycopersicum	S				S	S
Solanum demissum × *Aquila* (A-6)	L			L	L	S

Table 3

Indicator plants for detection of potato viruses (continued)

Species	Viruses					
	AMV	PAMV	TBRV	CMV	PMTV	TRV
Capsicum annum		L/S*				
Chenopodium album						
Chenopodium quinoa	L/S		LS*	L	L	L
Chenopodium amaranticolor	L/S		LS*	L	L	L
Datura metel						
Datura stramonium						
Gomphrena globosa	L/S*					
Lycopersicon chilense						
Nicotiana benthamiana	L/S			S	L	L
Nicotiana clevelandii			LS			
Nicotiana debneyi	S				L	L
Nicotiana glutinosa						
Nicotiana tabacum cv Samsun				L		L/S*
Nicotiana occidentalis P-1			LS	L?S		
Phaseolus vulgaris	L?S?					L*
Physalis floridana						
Solanum rostratum						
Solanum lycopersicum		L?	LS			
Solanum demissum × *Aquila* (A-6)						

Based on Muchalski and Chrzanowska (2001) with modifications; L – local symptoms; S – systemic symptoms; L/S – local and systemic symptoms; * - the most characteristic reaction of a given virus; ? Variable symptoms;
PVY *Potato virus Y*. PVM *Potato virus M*. PVS *Potato virus S*. PLRV *Potato leafroll virus*. PVA *Potato virus A*. PVX *Potato virus X*. PAMV *Potato aucuba mosaic virus*. TBRV *Tomato black ring virus*. AMV *Alfalfa mosaic virus*. CMV *Cucumber mosaic virus*. TRV *Tobacco rattle virus*. PMTV *Potato mop-top virus*.

PVY (*Potato virus Y*). PVY symptoms in the potato include mild and severe mosaic (Photo 1D), rugosity, crinkling, dropping of leaves and severe systemic necrosis (Photo 1B) and dwarfing. PVY^{NTN} isolates cause potato tuber necrotic ringspot disease (PTNRD) (Photo 1C) and necrotic foliar symptoms (Jeffries, 1998). The bioassay for PVY was conducted according to Singh *et al.* (2008). The potato differential cultivars used are cvs. King Edward, Desiree and Pentland Ivory supplemented by *Nicotiana tabacum* cv. Samsun. In addition, at Młochów Research Center, potato cv. Nicola is used for assessing PTNRD. PVY strain classification is based on its ability to elicit hypersensitive resistance (HR) mediated by *N* genes in differential potato cultivars. The PVY strains that elicit HR genes *Ny*, *Nc* and *Nz* are classified as PVY^{O}, PVY^{C} and PVY^{Z} strains, respectively. The PVY strains that overcome all these three HR genes are classified as PVY^{N} if they cause veinal necrosis (VN) in the tobacco (Photo 1A), or PVY^{E} if they do not induce VN in the tobacco. PVY^{N-Wi} and PVY^{NTN} belong to the PVY^{N} strain group, and PVY^{NTN} elicits PTNRD in sensitive potato cultivars. In addition, potato cultivars Etola and Gwiazda are used as the indicator for HR (Yin *et al.*, 2017).

PVM (*Potato virus M*). Based on the nucleotide sequences of the coat protein (CP) genes of PVM isolates from different countries (e.g., KC129086 to KC129101, JN835299, HM854296, EF063387, AY311194, AJ437481), all known PVM isolates fall into two distinct groups, i.e., PVM-ordinary (PVM-o) and PVM-divergent (PVM-d) (Xu *et al.,* 2010, Tabasinejad *et al.,* 2014). Polish potato cultivars infected in the field are often symptomless irrespective of the pathogenicity of the PVM strain (Chrzanowska *et al.*, 2002). However, most cultivars show severe symptoms when a severe strain of PVM is spread in the field (Zagórska *et al.*, 2000). Photo 2 shows the symptoms in the potato, e.g., severe dwarfing, crinkling and rolling of leaves caused by a severe PVM strain named Uran. In the indicator plants, PVM causes strong malformation of shoot tops in *Lycopersicon chilense*, local necrotic spots and systemic VN in *Solanum rostratum*.

PLRV (*Potato leafroll virus*). PLRV symptoms in the potato include yellowing or pale color of the apical or upper leaves, upward rolling of leaflets and stunting (Photo 3). In tubers, PLRV may cause net necrosis in the tuber flesh (Douglas and Pavek, 1972). In the indicator plants, PLRV causes yellow spots and interveinal chlorosis in *Datura stramonium*, weak yellowing (chlorosis), malformation and diminution of leaf size in *Physalis floridana*.

PVS (*Potato virus S*). PVS has been split into two strains, Ordinary (PVS^O) and Andean (PVS^A), based on non-systemic and systemic infection in *Chenopodium quinoa*. In the potato, PVS^O is symptomless on the majority of cultivars, while PVS^A may cause severe symptoms (Jeffries, 1998). In the potato cv. Defender, PVS-infected plants are severely stunted and necrotic lesions lead to drying of leaves and leaf drop; the symptoms incited by PVS on the late blight resistant potato breeding line LBR4106 plants include mosaic, severe necrosis, and leaf malformation (Lin, 2012). In the indicator plants, PVS causes veinal clearing (VCl) in *Nicotiana debneyi*, pale rings and figures in *D. stramonium*. In detached leaves of *Solanum demissum Y*, the PVS causes dark green and brown necrotic ring spots (Chrzanowska and Waś, 1974).

PVX (*Potato virus X*). PVX may cause mosaic, mottles or tuber necrosis in certain potato cultivars (Jeffries 1998). In the indicator plants, the PVX causes local rings and systemic mosaic or necrotic patterns in *N. tabacum*, VCl in *D. stramonium*, local lesions with red bordering in *Gomphrena globosa*.

TRV (*Tobacco rattle virus*). Potato plants grown from TRV-infected tubers show distortion, stunting, yellow mottle in leaves and stem mottle (Jeffries, 1998). In tubers, the TRV causes superficial necrotic rings and internal tuber symptoms of "spraing" (Photo 4D). In the indicator plants, TRV causes big grey -brown lesions in the inoculated leaves at 4 dpi, a brown line pattern, necrosis and distortion in the upper leaves (Photo 4A) and stem necrosis in *N. tabacum*, local and/or systemic lesions in *Chenopodium amaranticolor* and *Ch. quinoa* (Photo 4B and 4C, Yin *et al.,* 2014). In detached leaves of *S. demissum Y*, the TRV causes dark brown necrotic rings and line patterns (Chrzanowska and Waś, 1974). If soil samples with a potential to carry TRV are necessary for testing, tobacco seedlings are planted into the soil samples and the symptoms are evaluated after 10-30 days.

PVA (*Potato virus A*). In the potato, PVA may cause mild mosaic, roughness of surface and wavy leaf margin or no symptoms depending on the cultivars (Jeffries, 1998). In the indicator plants, PVA causes VCl, diffuse mottle, dark-green vein-banding in the *N. tabacum* cv. Samsun.

PMTV (*Potato mop-top virus*). In potato tubers, a PMTV infection may cause slightly raised lines, rings, or brown arcs on the surface, and lines (spraing) in the flesh (Jeffries, 1998). Plants grown from the PMTV-infected tubers show yellow blotching or mottling on the lower leaves, chlorotic V-shaped markings (chevrons) in leaflets, and extreme stunting of the shoots (mop -top) (Jeffries, 1998). However, these symptoms have been confused with those caused by the *Alfalfa mosaic virus*, the *Potato aucuba mosaic virus*, TRV and the *Tomato black ring virus* (Jeffries, 1998). In the indicator plants, PMTV causes concentric fine necrotic ringspot lesions in the inoculated leaves at 7 dpi, but not systemic in *Ch. amaranticolor*, necrotic or chlorotic ringspots in local leaves and chlorotic or necrotic 'thistle-leaf' line patterns in systemic leaves in *N. debneyi* and *N. tabacum*.

TBRV (*Tomato black ring virus*). In the potato, TBRV may cause black necrotic rings or spots in leaves, the infected tubers may be cracked and distorted (Jeffries, 1998). In the indicator plants, TBRV causes chlorotic or necrotic local lesions, systemic necrosis or chlorotic mottle in *Ch. amaranticolor* and *Ch. quinoa*, local chlorotic or necrotic spots or rings, systemic spots, rings and line patterns with variable amounts of necrosis in *N. tabacum*.

AMV (*Alfalfa mosaic virus*). In the potato, AMV may cause calico symptoms of bright yellow blotching or mottling of leaflets, chlorosis to necrosis of leaflets, tuber necrosis, misshapen or cracked tubers (Jeffries, 1998). The tuber symptoms resemble those caused by the PMTV or TRV. In the indicator plants, AMV causes systemic yellowing of leaves in *Gomphrena globosa*.

PAMV (*Potato aucuba mosaic virus*). In the potato, PAMV may cause yellow spots, flecking, blotching, mottling and necrotic spots in leaves, top necrosis and stunting of plants. In tubers, external, internal and net necrosis may occur. The tuber surface symptoms may resemble those caused by PMTV (Jeffries 1998). In the indicator plants, PAMV may cause necrotic local lesions and systemic VCl and VN in *Capsicum annum* (Jeffries, 1998).

CMV (*Cucumber mosaic virus*). CMV may cause chlorosis, mottling and blistering of potato leaves (Jeffries, 1998). Chrzanowska *et al.* (2004) transmitted three CMV isolates to 27 potato cultivars by mechanical inoculation. Local chlorotic symptoms and systemic necrosis, mosaic and malformation of leaves were observed. In the indicator plants, CMV causes chlorotic or necrotic local lesions but no systemic infection in *Ch. amaranticolor* and *Ch. quinoa*, chlorotic spots and pattern in local and systemic leaves in *D. stramonium*, reddish local spots with white center, systemic reddish brown spots and yellow spots in the youngest leaves in *G. globosa*.

BMYV (*Beet mild yellowing virus*). BMYV causes mild yellowing of the sugar beet (Kozłowska-Makulska *et al.,* 2015). The virus is restricted to the phloem tissue. This virus is only maintained in the collection.

The infected plants with the characteristic symptoms for a given virus are subjected to serological and molecular assays.

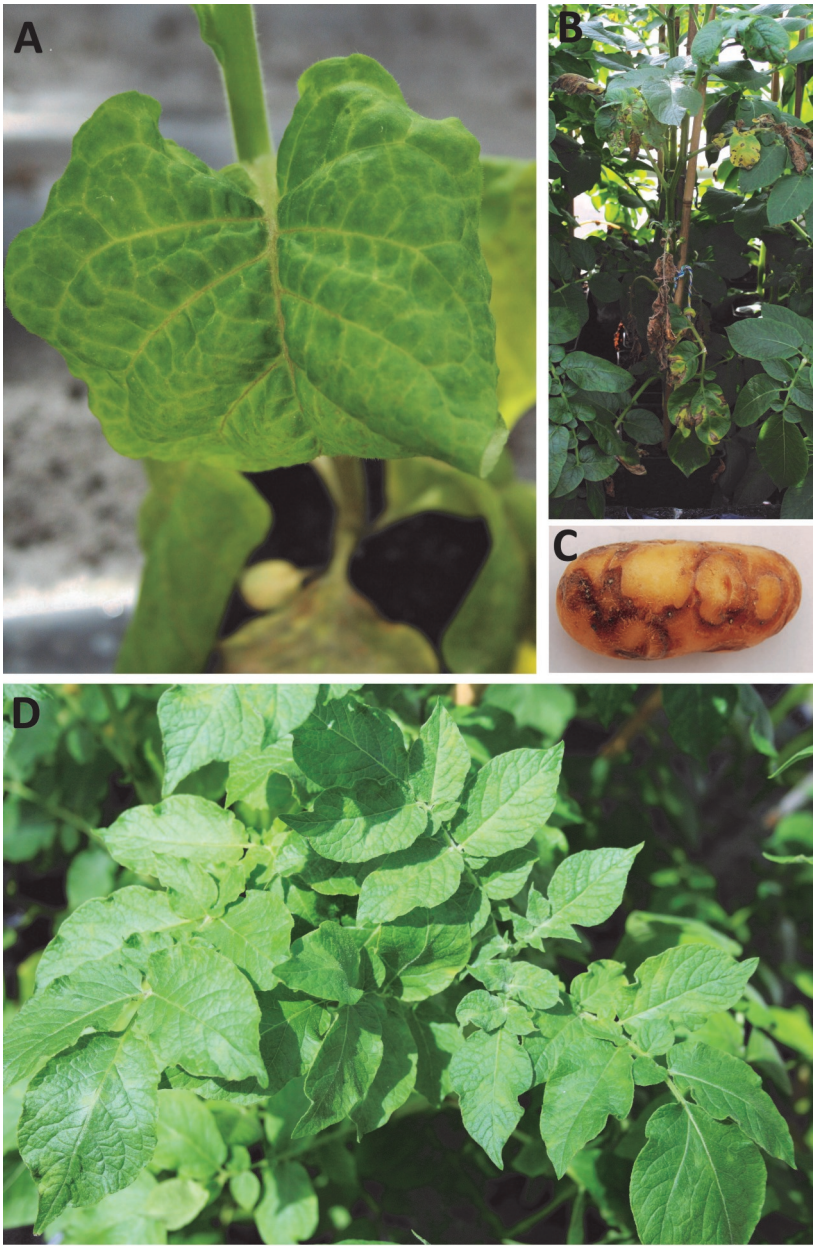

Photo 1. Disease symptoms caused by PVY infection. A: Veinal necrosis (VN) in the tobacco cv. Samsun caused by PVY^{NTN} infection. B: Severe systemic necrosis and dropping of leaves caused by PVY^{NTN} in potato cv. Gwiazda. C: Severe superficial tuber necrosis (potato tuber necrotic ringspot disease - PTNRD) caused by PVY^{NTN} in the potato cv. Nicola. D: Mosaic in the potato cv. Lady Claire caused by $PVY^{N\text{-}Wi}$ infection.

Photo 2. Dwarfing, crinkling and rolling of leaves caused by a severe PVM isolate named Uran in the potato

Photo 3. Stunting, yellowing and upward rolling of leaves caused by a PLRV isolate named L_7 in the potato cv. Osa

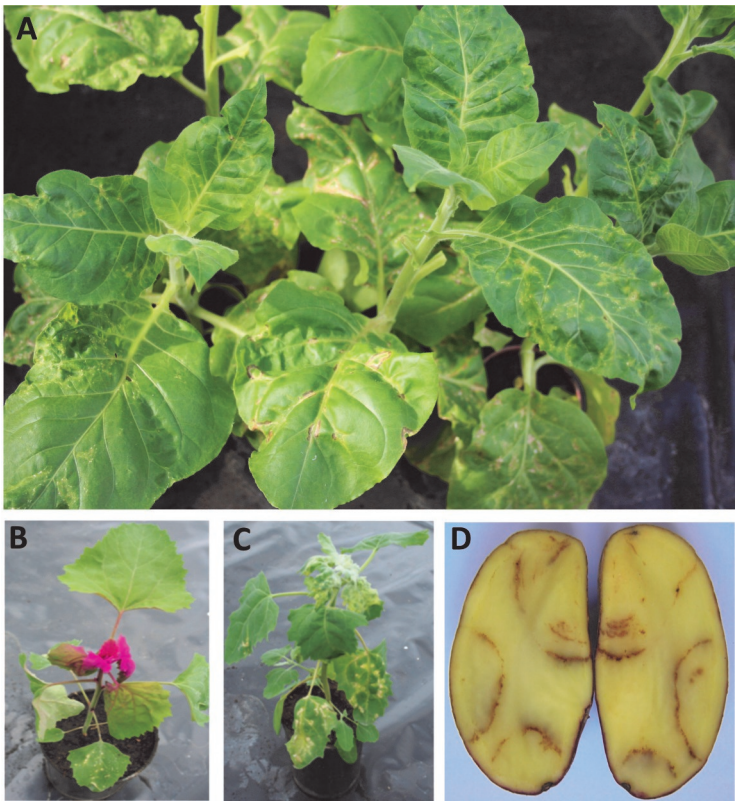

Photo 4. Disease symptoms caused by TRV. A: Systemic distortions, necrotic lesions and line patterns on the leaves and lesions on the stems in the tobacco cv. Samsun. B: Local lesions and severe systemic leaf distortions in *Chenopodium amaranticolor*. C: Local lesions and systemic leaf distortions and necroses on *Chenopodium quinoa*. D: Internal tuber symptoms of "spraing" in the potato cv. Elanda.

Serological assay – ELISA

1) The serological test is conducted when the virus-infected plants show characteristic symptoms. Leaves with potential symptoms are taken from the plants.
2) Leaf sap was extracted by passing through a squeezing machine (Press for squeezing leaves, producer: SUDD-ELECTROMOTORENWERKE BRUCHSAL, Type DFWZ-1B-1-2, No 12408290) and mixed with an extraction buffer in 1:1 ratio, and 200 µl of the leaf extract are loaded into a 96-well plate.
3) ELISA tests using polyclonal or monoclonal antibodies can be conducted for detection of PVY, PVA, PVM, PVS, PLRV, PVX, TRV and PMTV.
4) The ELISA procedure is according to Syller (2001).
   a) Add 200 µl of γ-globulin diluted in coating buffer to each well of the plate.
   b) Incubation 2-4 h at 37°C.
   c) Remove γ-globulin solution and wash the plate very carefully 3 times.
   d) Add 200 µl of plant extract diluted in extraction buffer to each well.
   e) Incubation overnight at 4°C.
   f) Remove plant extracts and wash the plate carefully 3-4 times.
   g) Add 200 µl conjugate, diluted in conjugate buffer to each well.
   h) Incubation for 2-4 h at 37°C.
   i) Remove conjugate and wash the plate carefully 3 times.
   j) Add 200 µl of freshly prepared enzyme substrate solution (0.75-1.0 mg per 1 ml of substrate buffer).
   k) Incubation at room temperature for 0.5 to 2 h.
   l) Read the absorbance values for each well at 405 nm with the ELISA reader (DYNATEX MRX II).

Molecular assay – RT-PCR (Reverse transcription polymerase chain reaction), multiplex RT-PCR, real-time RT-qPCR and sequencing

The conventional simplex or multiplex RT-PCR is used for detecting PVY, TRV and PMTV.

The RT-PCR procedures can be used for detecting other potato viruses with virus-specific primers. The source for the available primers from the published data is summarized in Table 4. In addition, a method for PVY and TRV whole-genome sequencing and real-time quantitative RT-PCR (real-time RT-qPCR) detection of PVY *HC-Pro* RNA are referred to in Yin *et al.* (2014, 2017), and the primer sequences are listed in Table 5. The primer sequences used for detecting of TRV and PMTV by RT-PCR are shown in Table 6.

1) RNA extraction from plant tissue (leaves, tubers)
   a) The total RNA from plant tissue is extracted by using the RNeasy Plant Mini kit (Qiagen, cat. No. 74904) according to the manufacturer's instructions.

b) Briefly, extract 100 mg of tissue powder with 450 µl of RLT (applied in the kit) buffer. Add 0.5 volume of ethanol (96-100%) to the clear lysate.
c) Bind the RNA to the RNeasy spin column and wash by RW1 and PRE (applied in the kit) buffer.
d) Elute the RNA in 60 µl of RNase-free water. Store the RNA at – 80°C.

2) RT-PCR / multiplex RT-PCR
a) RT-PCR is performed using Superscript III one-step RT-PCR with Platinum Taq DNA polymerase (Invitrogen, cat. No 12574026).
b) In brief, a 25 µl reaction mix consists of 100-1000 ng RNA template, 12.5 µl of 2x reaction buffer, sense and antisense primer at a final concentration 0.2 µM, 1 µl of superscript III RT / Platinum Taq Mix (applied in the kit) and Rnase-free water.
c) In the case of multiplex RT-PCR, multiple pairs of primers targeting specific viruses or virus strains are used.
d) The amplification program is as follows: 1 cycle 54°C, 25 minutes; 1 cycle 94°C, 2 min; 30 cycles of 94°C, 20 s, 57°C, 20 s, and 68°C, 1 min; 1 cycle 68°C, 5 min.
e) The RT-PCR products were visualized on 1.5% agarose gel by ethidium bromide staining.

3) Real-time quantitative RT-PCR (Real-time RT-qPCR)
a) Reverse transcription is performed using the TaqMan micro-RNA Reverse Transcription kit (Applied Biosystems, cat. No. 4366597) according to the manufacturer's instructions.
b) A total reaction volume of 15 µl containing 1 µg of RNA and 0.2 µM virus-specific reverse primers is run using the GeneAmp PCR System 9700 (Applied Biosystems).
c) The reverse transcription parameters are: 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, followed by a hold at 4°C.
d) Real-time quantitative PCR (qPCR) was performed in 96-well plates with a LightCycler 480 real-time PCR instrument (Roche Diagnostics) using the SYBR Select Master Mix (Applied Biosystems, cat. No 4472908) as described by the manufacturer.
e) Briefly, each reaction was performed in a 10 µl reaction mixture containing 3 µl of Dnase/ Rnase-free water (applied in the kit), 5 µl of the SYBR Select Master Mix, 1 µl of diluted cDNA, and 2 µM of each of the gene-specific forward and reverse primers.
f) The cycling conditions were as follows: UDG activation at 50°C for 2 min and AmpliTaq Fast DNA polymerase and UP activation at 95°C for 2 min, followed by 40 cycles of denature 95°C for 10 s and anneal/extend 60°C for 1 min. All the assays included no-template controls to verify nonspecific amplification.
g) The primer sequences used for detecting PVY, TRV and PMTV are listed in Table 5 and 6. For detecting viruses in potato, the reference genes used are the potato β-tubulin gene (Z33402).

4) Sequencing
   a) Overlapping RT-PCR fragments are amplified using the RNA extracted from a virus-infected plant using specific primers.
   b) The RT-PCR products are purified by QIAquick purification kit (Qiagen, cat. No 28104) and sequenced directly by the DNA Sequencing Laboratory, IBB, PAS, Poland, using MiSeq sequencer (Illumina).
   c) Sequence ambiguities are resolved by Sanger sequencing. Multiple sequence alignments are obtained using CLUSTALW and MEGA v. 6 (http://www.megasoftware.net).

Table 4

The published reference list in which the virus-specific or strain-specific primers are available

Virus	Detection method			References
	I	II	III and IV	
TRV	RT-PCR			Heinze *et al*., 2000
AMV	RT-PCR			Xu and Nie, 2006
PVM	RT-PCR			Xu *et al.*, 2010
PMTV	RT-PCR			Santala *et al*., 2010
TBRV	RT-PCR			Harper *et al*., 2011
PLRV, PVA, PVS, PVX, PVY		Multiplex RT-PCR		Nie and Singh, 2001
PLRV,PVA, PVS, PVX, PVY		Multiplex RT-PCR		Du *et al*., 2006
PVY		Multiplex RT-PCR		Lorenzen *et al*., 2006 Rigotti and Gugerli, 2007 Chikh Ali *et al*., 2010
PLRV, PVS, PVX		Multiplex RT-PCR		Peiman and Xie, 2006
AMV, PLRV, PMTV, PVA, PVM, PVS, PVX, PVY, TRV		Multiplex RT-PCR		Crosslin and Hamlin, 2011
CMV		Multiplex RT-PCR		Panno *et al.,* 2012
PAMV, PLRV, PVM, PVS, PVX		Multiplex RT-PCR		Kumar *et al., 2017*
PVY			Real-time RT-qPCR, Sequencing	Yin *et al*., 2017
TRV			Sequencing	Yin *et al.,* 2014
TRV			Real-time RT-qPCR	Mumford *et al*., 2000
BMYV*		Multiplex RT-PCR		Hauser *et al.,* 2000

See footnote in Table 2. * BMYV *Beet mild yellowing virus*. BMYV is maintained in the collection only.

Table 5

Primer sequences used for the whole genome sequencing of PVY and TRV and real-time RT-qPCR detection of PVY

(a) PVY[a]			
Overlapping fragment	Primer	Sequence (5' → 3')	References
A	70Ya1f1[b]	AAATTAAAACAACTCAATACAACATAAGAA	Yin *et al.* 2017
	70Ya2r3497	GTGTCGCACATCATATTCTTCCA	
B	70Yb1f3122	CTCAGGATTGAGCGAGCGAT	
	70Yb2r6562	GGGAAAACAGGGAAATCCTTTGG	
C	70Yc1f6221	TGGGCCAGCAATAGAGGTTG	
	70Yc2r9697	CTCCTGATTGAAGTTTACAGTCACT	
PVY-3411 B[c]	o6400 (reverse)	GTAACTCCTAAACAAATGGTGGTTCG	Chikh-Ali *et al.* 2010
PVY-3411 C[d]	S5585m (forward)	GGATCTCAAGTTGAAGGGGAC	Lorenzen *et al.* 2006
(b) TRV			
TRV RNA1	r1-2 (forward)	ATAAAACATTTCAATCCTTTGAACG	Crosslin *et al.* 2010
	r1-1 (reverse)	GGGCGTAATAACGCTTACGTAG	
TRV RNA2	R2-4 (forward)	ATAAAACATTGCACCWWTGGTGTTGC	Crosslin *et al.* 2003
	R2-3 (reverse)	CGTAATAACGCTTACGTAGGCGAG	
(c) real-time RT-qPCR detection of PVY			
PVY HC-Pro	Forward	GAATCAACATTTTACCCGCCAACT	Yin *et al.* 2017
	Reverse	GCACACACATGTCACGAACCTTCTTAG	
β-TUB (reference gene)	Forward	TGTTTGGACAGTCTGGTGCTGG	Z33402 Yin *et al.* 2017
	Reverse	TCACAATTCTCCGCTTCTTTACG	

[a]Primer sequences were designed based on PVY isolate 12-94 (AJ889866); [b]Naming of primer: r indicates reverse primer, f indicates forward primer, nucleotide position was indicated at the end; [c]Forward 70Yb1f3122 and reverse o6400 primers were used for amplification the fragment B of PVY-3411 (KX356070); [d]Forward S5585m and reverse 70Yc2r9697 primers were used for amplification the fragment C of PVY-3411 (KX356070); PVY HC-Pro: potato virus Y helper-component proteinase. β-TUB: β-tubulin. PVY: potato virus Y. TRV: tobacco rattler virus.

Table 6

Oligonucleotide primers used in RT-PCR detection of PMTV and TRV at IHAR-PIB/Młochów

(a) Type of primers	Symbol of primers	Locations	Sequence	References
PMTV	F1Y	RNA1, RdRp 5502 – 5522 pos.	5'- CCA TAA AGG CAG GTA CCA GCG -3'	AJ238607
	R1Y	RNA 1 5852 – 5872 neg.	5'- GTG AAC CAC GGT TTA RCC CTG -3'	
	Fpr25	RNA2 RT-mid 1154-1174 pos.	5'-GGA CTT CCT TGG CTA TTA GCG-3'	AJ243719
	Rpr24	RNA2 RT-mid 1518-1539 neg.	5'-TCC CTG TCA CCT TCA TCT ATG G-3'	
	CP1	RNA2 CP 292-311 pos.	5'-GGT TTG TTT ACC ACC CTT GG-3'	
	8Kfor	RNA3, 8k 2421-2439 pos.	5'-GTA GCA AGT ACG CCC TGT G-3'	AJ277556
	3'rev	3'-end RNA2, 3114 -3134 neg. 3'-end RNA3, 2944- 2964 neg.	5'-TGG TCT TGG ATA CCC TCC AAG-3'	
TRV	TRV-W2	RNA1, 16 k 6115 – 61331 pos.	5'-CGT GTG TAC TCA AGG GTT-3'	NC 003805, Heinze *et al.* 2000
	TRV-180	RNA1 16 k 6768 – 6791 neg.	5'-TCT AGA GGG CGT AAT AAC GCT TAC-3'	
Internal control	NAD5 F		5'-CTC CAG TCA CCA ACA TTG GCA TAA-3'	Menzel *et al.* 2002
	NAD5 R		5'-CTC CAG TCA CCA ACA TTG GCA TAA-3'	

(b) Tested virus	Primer pairs	Targeted sequence	Expected band
PMTV	[a]F1Y + R1Y	PMTV RNA1 RdRp	371 bp
	[b]Fpr25 + 3'rev	PMTV RNA2 RT	1981 bp
	[b]CP1 + Rpr24	PMTV RNA2 CP	1248 bp
	[b]8Kfor + 3'rev	PMTV RNA3 8k	544 bp
TRV	TRV-W2 + TRV-180	TRV RNA1 16k	677 bp
Internal control	NAD5 F + NAD5 R		180 bp

[a]Primer sequences designed and used at IHAR-PIB/Młochów; [b]Primer sequences according to the project "Enhanced control of *Potato mop-top virus* in the Nordic and Baltic Sea region" (Santala *et al.* 2010); RT: readthrough domain. RdRp: RNA-dependent RNA polymerases. CP: coat protein. 8k: 8k protein . 16k: 16k protein.

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