Pestic. Phytomed. (Belgrade), 30(1), 2015, 17–24
DOI: 10.2298/PIF1501017M

UDC 632.38:635.21:58:083
Original scientific paper

Potato cv. Romano reaction to primary and secondary infection with potato necrotic strain Y virus (PVY^{NTN})

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Received: December 23, 2014
Accepted: February 13, 2015

SUMMARY

Primary and secondary infections with PVY^{NTN} were investigated on forty plants of the potato cv. Romano inoculated in a greenhouse in Serbia in 2012 and 2013. PVY isolates were collected from the potato growing region of Čačak and identified by ELISA and RT-PCR methods. The sequence of the Serbian isolate 3D (Acc. No. KJ946936) showed 100% match with seven PVY isolates deposited in GenBank and described as NTN. A significant difference was detected between PVY^{NTN} symptoms exibited on leaves of the cv. Romano under primary and secondary infections. The findings are significant because they are based on symptoms observed, so that it is clear that there are two distinct types of infection: primary and secondary. Symptoms of primary and secondary infection were the same on potato tubers and had the form of necrotic rings.

Keywords: Potatoes; Plant viruses; Potato Y potyvirus; Infection

INTRODUCTION

*Potato virus Y (*PVY) belongs to the lagest plant virus family *Potyviridae*. PVY naturally infects plants from more than 9 families, including 14 genera of the *Solanaceae* family, such as potato, pepper, tobacco and tomato (Shukla et al., 1994; Kerlan, 2006). The virus significantly reduces potato yield and is the most important limiting factor for seed potato production in Serbia and neighbouring countries (Milošević, 2009). In terms of distribution and speed of infection of healthy potato plants in Serbia, PVY is the most important virus, followed by *Potato leafroll virus* (PLRV) and *Potato virus S* (PVS) (Milošević, 1992, 1996; Gavran, 1997; Milošević et al., 2000; Milošević, 2009, 2013). The most dominant strain of PVY is the necrotic strain, which is able to infect more than 50% of plants in a crop, depending on location and potato cultivar. The occurrence and rate of infection with PVY has been studied over three decades and its distribution has become epidemic in Serbia and its wider region (Milošević, 2013). Potato viruses are economically the most important potato pathogens, causing most damage in potato production (Buturović & Kus, 1990; Kus & Hočevar, 1977; Dolničar, 2004; Zindović, 2011).

In addition to yield reduction due to infected potato tubers, the PVY^{N}/PVY^{NTN} necrotic strain causes also the potato tuber necrotic ringspot disease (PTNRD), which infects the potato cultivar Romano, resulting in

its lower market value (Milošević, 1994a,b; Milošević et al., 2008, 2011). Over the past several years since its introduction in Serbia, it has been noted that cv. Romano is susceptible to the ringspot necrosis of tubers. The epidemic nature of PVY^{NTN} infection has contributed to the frequency of tuber ringspot necrosis on all susceptible potato genotypes (Milošević, 1992, 2013; le Romancer & Nedellec, 1997; Varveri & Bem, 2001).

PVY^{NTN}, the causal agent of potato tuber necrotic ringspot disease, was detected in a number of European countries in the 1980s (Beczner et al., 1984; Schiessendoppler, 1990; Schiessendoppler & Rauscher, 1999; McDonalds & Singh, 1993; Bem et al., 1999). First symptoms were detected on the Monalisa cultivar in Hungary (Beczner et al., 1984), then in other regions of Europe (Bem et al., 1999; Blanco-Urgoiti et al., 1998; Buturović & Kus, 1989; Kus, 1995; le Romancer & Nedellec, 1997; Serra & Weidemann, 1997), and finally spread worldwide – in North and South America (McDonald & Singh, 1996; Salazar et al., 2000), New Zeland *(Hay, et al., 1989) and Japan (Ohshima et al., 2000).

In the region of former Yugoslavia, the potato tuber necrotic ringspot disease was first detected on potato tubers in Bosnia and Hercegovina in 1987 (Buturović & Kus, 1989), and then in Slovenia and Serbia in 1989 (Buturović & Kus, 1990; Horvath et al., 1997; Milošević, 1994b). Over the following years, potato tubers with such symptoms were observed on many cultivars and in different localities in Serbia. As extreme susceptibility was detected in preliminary experiments, the following cultivars have been recommended for exclusion from further cultivation in Serbia: Igor, Arielle, Finka, Hermes, Colette, Inova, Gala, Caruso and Saphire (Milošević, 1994a,b; Milošević et al., 2008; Milošević et al., 2011).

The symptoms of PTNRD and other necrotic symptoms include characteristic rings. They are superficial, shallow or slightly deeper but always on the tuber surface. This study focused on investigating the primary and secondary PVY infection on potato leaves and tubers of the cultivar Romano, and their differentiation, performing its first molecular identification in Serbia.

MATERIALS AND METHODS

Isolation. Virus samples were collected from symptomatic potato plants cv. Romano in the locality Čačak in 2012. The infected potato plants and inoculated *Nicotiana tabacum* L. cv. Samsun test plants were cultivated in sterile soil in a greenhouse.

Serological tests. Samples were analysed by DAS-ELISA (Clark & Adams, 1977) using a commercial polyclonal antibody (Bioreba AG) for detecting the presence of PVY, PLRV, *Potato virus A* (PVA), *Potato virus M* (PVM), *Potato virus S* (PVS) and *Potato virus X* (PVX). In PVY detection, the antibodies for detection of all PVY strains and those for only the necrotic strain (PVY^{N}) were used. Samples with A405 readings three-fold higher than those of healthy controls were considered as infected.

RNA extraction and RT-PCR. Total RNA was extracted from infected potato tubers using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and following the manufacturer's instructions. Total RNA was diluted in 50 µl of RNase free water and centrifuged for 1 min at 10000 rpm. The resulting RNA was stored at -80°C.

Four microliters of concentrated total RNA extract was diluted with 6 µl of RNase free water to reduce nonspecific amplification (Singh et al., 2003), incubated at 70°C for 5 min and chilled on ice for 3 min. To the denatured RNA extract, 15 µl of RT reaction mixture Oligo-dT Primer, 5x FS Buffer, 40 units of RiboLock RNase Inhibitor, 10 mM dNTP and 200 units of M-MLV reverse transcriptase (Invitrogen, CA, USA) were added to provide a final volume of 25 µl. The samples were incubated at 42°C for 1 h, followed by 75°C for 5 min to terminate the RT reaction.

PCR was performed using the primer pairs CPfwd (5'-ACCATCAAGSAAATGACACA-3') and CPrev (5'-CGGAGAGACACTACATCACA-3'), which flank the conserved fragment of the coat protein gene located on RNA, as described in Glais et al. (2002). The cDNA (1 µl) product was added to a 24 µl PCR reaction mixture consisting of 12.5 µl 2X Green Master Mix (Promega, Madison, WI, USA), 1.25 µl each of both forward and reverse primers (100 pmol/µl, Invitrogen, CA, USA) and 9 µl RNase-free water. The mixture was subjected to 30 cycles of heating and cooling in a 2720 Thermal Cycler (Applied Biosystems). Each cycle consisted of 1 min at 94°C for denaturation, 1 min at 57°C for primer annealing and 1 min at 72°C for primer extension, followed by a 10 min final extension at 72°C. Amplified products were analyzed by 1% agarose gel electrophoresis, stained with Midori Green DNA Stain (Nippon Genetics), and visualized under a UV transilluminator.

Sequence analyses. Sequencing in both directions was performed on an automated sequencer (ABI 3730XL Automatic Sequencer Macrogen, Korea). All sequences generated in this study were deposited in the National Center of Biotechnology Information (NCBI) GenBank database. Sequences of the Serbian virus isolates were

compared with the respective virus sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using the ClustalW program (Thompson et al., 1994) and MEGA5 software (Tamura et al., 2011). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) sequence analyses and the divergence of selected virus isolate sequences was calculated using sequences trimmed to the length of the shortest fragment.

Inoculation. Eighty healthy tubers of the susceptible cv. Romano "basic" category were selected. They were grown in sterile soil in the greenhouse with temperatures between 25 and 30°C for 15-18 h during daytime and 15 to 20°C during nighttime. Testing for the presence of PVY, *Potato leafroll virus* (PLRV), *Potato virus A* (PVA), *Potato virus M* (PVM) and PVS was run by ELISA test using monoclonal antibodies (IgG) specifically needed to detect the necrotic strain, as well as antibodies needed to detect all other strains of PVY. After the ELISA results proved negative, 40 plants were infected with the strain Y-VKr ^{NTN} 15 days after germination, and another 40 plants were used as a negative control. Inoculation was carried out with infected tobacco plants (*Nicotiana tabacum* cv. Samsun) in a phosphate buffer using air under pressure, i.e. „air brush". Fifteen days after inoculation, ELISA test was performed again to detect the presence of PVY. Table 1 shows the results of PVY activity in plants before and after test inoculation.

Twenty-five days after inoculation, visual inspection was carried out to detect symptoms on potato leaves, as well as ringspot necrosis on harvested tubers. During the following spring, 40 tubers were planted, 37 demonstrating symptoms of ringspot necrosis and 3 without any symptoms, in order to detect possible secondary symptoms on the harvested leaves and tubers.

RESULTS AND DISCUSSION

Detection and identification of PVY isolate. RT-PCR products of 826-bp were obtained from all samples that were serologically positive to PVY, while no amplicon was recorded in the healthy control. In a BLAST search analysis, the sequence of the Serbian isolate 3D (Acc. No. KJ946936) showed the highest nt homology of 100% (100% aa identity) with seven PVY isolates deposited in GenBank, of which three isolates were described as NTN strains from South Africa (Acc. No. JN936430-32).

Primary symptoms of PVY infection. Thirteen days after inoculation in the greenhouse, first symptoms showed on the leaves of inoculated potato plants. All inoculated plants reacted positively to the ELISA test using mono- and polyclonal antibodies for PVY detection. Control plants showed a negative result to ELISA tests. ELISA test results for the presence of a PVY necrotic strain before and after inoculation are presented in Table 1.

Table 1. Results of ELISA test: Virus detection in inoculated and control plants 15 days after inoculation

	Inoculated plants							Control (noninoculated plants)					
	PVY^{P}	PVY^{M}	PLRV	PVA	PVM	PVX	PVS	PVY^{P}	PLRV	PVA	PVM	PVX	PVS
BI	**40^{A}/0^{B}**	**40/0**	40/0	40/0	40/0	40/0	40/0	40/0	40/0	40/0	40/0	40/0	40/0
AI	**40/40**	**40/40**	40/0	40/0	40/0	40/0	40/0	–	–	–	–	–	–

BI – ELISA test before inoculation
AI – ELISA test after inoculation
^{P} Polyclonal antibody for detection of all PVY strains
^{M} Monoclonal antibody for detection of necrotic PVY only
A/B – Tested / infected plants

Table 2. Symptoms induced by PVY^{NTN} in potato cv. Romano

Origin of infection	Symptoms*				
	Young leaves		Mature leaves		Tubers
	face	down	face	down	
Primary	VN, RN	RN, DN	VN, RN, Y,D	RN, DN, Y,D	PN
Secondary	M, Mo, B		M, Mo, B		PN

*VN – vein necrosis
RN – ring necrosis
DN – dotted necrosis
Y – yellowing
D – decline
M – mosaic
Mo – motttling
B – banding
PN – tuber ringspot necrosis

Plants inoculated with the necrotic strain Y virus exhibited symptoms of ringspot necrosis and vein necrosis on leaves (Figure 1a). Symptoms of ringspot and spotted necrosis also showed on leaf undersides. The symptoms were similar on mature leaves. In addition to the oldest, other low-growing leaves also became yellow, dried and dropped off. The symptoms on potato tubers were necrotic rings (Figure 2).

Secondary symptoms of PVY infection. The first inspection of symptoms was made 15 days after germination of potato plants. Secondary symptoms included mosaic, spotting and curling of leaves (Table 2, Figure 1 b,c). Symptoms were found also on the plants sprung from tubers without symptoms originating from infected plants.

The second inspection was carried out 10 days after the first (25 days after germination). Secondary symptoms were the same as before. Potato tubers of the secondary infected plants of cv. Romano showed symptoms of PTNRD identical to primary infection (Figure 2). All healthy plants were without symptoms.

Although PVY has already been reported as an economically important plant pathogen with a wide host range that includes potato, tobacco, tomato and pepper crops in Serbia (Milošević et al., 2008, 2011), there are no data on molecular identification of domestic PVY strains. This investigation showed that the sequences of Serbian PVY isolates were highly homologous to the CP gene sequences of PVY isolates from other parts of the world. Previous studies had shown a high degree of CP gene sequence identity of PVY isolates of many potato cultivars from Ukraine (Budzanivska et al., 2014).

Figure 1. Foliar symptoms on potato cv. Romano: (a) primary infection: vein necrosis, ring necrosis and dotted necrosis on lower leaves; (b, c) secondary infection: mosaic, mottling – left: healthy plant, right: infected plant

A significant difference was found between the exibited PVY^{NTN} symptoms on leaves of cv. Romano plants under primary and secondary infection. The exhibited symptoms of primary infection in these essays included ringspot necrosis and vein necrosis, as well as ringspot and spotted necrosis. The symptoms of secondary infection were mosaic, spotting and curling of leaves. These data are very important because they are based on the symptoms displayed and therefore lead to a clear conclusion that there are two distinct forms of infection: primary and secondary. The symptoms of necrotic rings on potato tubers were similar in both primary and secondary infection.

Baldauf et al. (2006) and Ramírez-Rodríguez et al. (2009) previously described symptoms of the PVY – necrotic strain as mosaic on leaves and ringspot necrosis on tubers. The development of symptoms depends also on temperature in the field and place of storage, and on cultivar (le Romancer & Nedellec, 1997). The necrotic strain causes mosaic symptoms on leaves in most cultivars (de Bokx & Huttinga, 1981). Kerlan et al. (1999) and le Romancer et al. (1994) had shown that PVY^{NTN} isolates caused PTNR. PVY^{NTN}, which belongs to PVY^{N}, only causes symptoms of ringspot necrosis on tubers (Hu et al., 2011). PVY^{N} and PVY^{O} have been shown to have recombined to the form PVY^{NTN} that causes PTNR (Boonham et al., 1999, 2002; Gray et al., 2010; Ohshima et al., 2000; Singh et al., 2008). Subsequent biological characterization of PVY^{NTN} isolates on cvs. Desiree and King Edward showed induced systemic tuber necrosis (Kerlan et al, 2011).-

Baldauf et al. (2006) found PVY^{NTN} to cause ringspot necrosis of potato tubers, while PVY^{O}, PVY^{C}, PVY^{N} and $PVY^{N:O}$ did not. Whitworth et al. (2011) reported that some potato cultivars were susceptible and some resistant to PTNR and that environmental conditions could either stimulate or inhibit the intensity of symptoms. They found that $PVY^{N:O}$ and PVY^{NTN} caused PTNR symptoms, while PVY^{O} did not produce any symptoms (except in a single isolate - T1). Cerato et al. (1999) reported from Italy that the ultivar Hermes was susceptible to PVY^{NTN} (PTNR). Schiessendoppler and Rauscher (1999) found

that the percentage of tubers with PTNR symptoms was much lower than the percentage of PVY^{NTN}-infected tubers. Sometimes it is only a single tuber that exibits symptoms, while others carry a latent infection. This fact has been confirmed in our research and we found that tubers from infected plants without symptoms were able to transmit infection and produce infected plants. Hu et al. (2009) found that only PVY^{NTN} produced PTNR symptoms, while PVY^{O} and $PVY^{N:O}$ did not.

Based on the results of our investigation of PVY isolates and their effects on the intensity of symptoms on leaves and tubers of the potato cultivar Romano, the following conclusions can be inferred:

1. The isolate that inoculated healthy plants of the potato cv. Romano belongs to the necrotic strain of potato virus Y (PVY^{NTN}).

2. Primary symptoms were caused by that PVY isolate on leaves of the potato cv. Romano and manifested as ring and punctate necrosis of leaves.

3. Secondary symptoms took the form of mosaics, and colorful and banding leaves.

4. Tuber ring necrosis (PTNR) was a symptom observed on tubers in both primary and secondary infection.

5. PTNR symptoms were not found on any tubers of uninfected plants.

6. Tubers without symptoms originating from infected plants can transmit the disease.

ACKNOWLEDGEMENT

This study was part of the project "Research into plant pathogens, arthropods, weeds and pesticides towards the development of biorational crop management methods and safe food production" (TR31043), funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

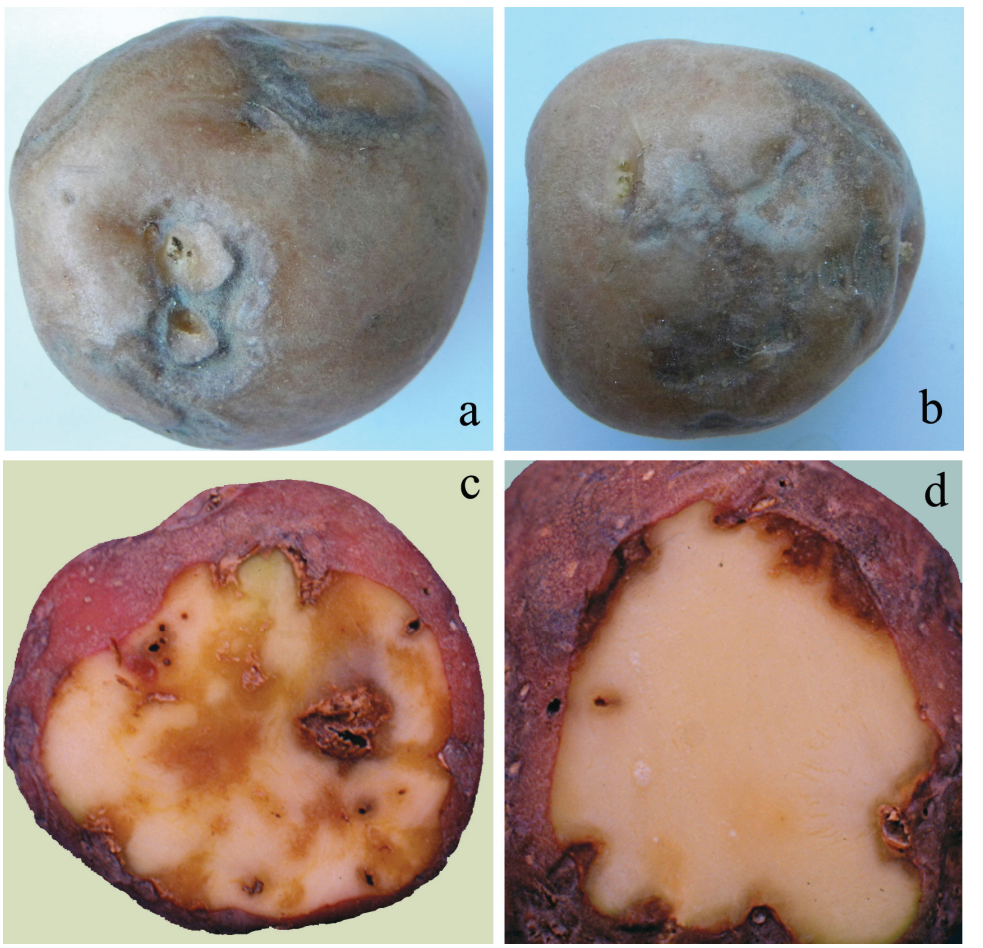

Figure 2. Symptoms on potato tubers cv. Romano (a,b) on the surface; (c,d) inside tuber

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Reakcija sorte Romano na primarne i sekundarne zaraze nekrotičnim sojem Y virusa krompira (PVY^{NTN})

REZIME

Upoređivani su simptomi primarnih i sekundarnih zaraza na četrdeset biljaka sorte Romano inokulisanih izolatom PVY^{NTN} u uslovima staklare, tokom 2012 i 2013 godine. Izolat PVY je kolekcionisan u lokalitetu Čačak, identifikovan je ELISA testom i okarakterisan RT-PCR metodom kao PVY^{NTN} soj. Sekvenca CP gena odabranog izolata 3D (Acc. No. KJ946936) ispoljila je 100% nukleotidnu identičnost sa sedam sekvenci PVY izolata deponovanih u GenBank opisanih kao NTN soj. Ispoljeni simptomi na biljnoj masi primarno i sekundarno zaraženih biljaka opisanim sojem su značajno različiti. Na osnovu ovih razlika može se zaključivati da li su se biljke zarazile u tekućoj godini ili su sađene već zaražene krtole. Prstenasta nekroza je zajednički simptom na krtolama primarno i sekundarno zaraženih biljaka.

Ključne reči: Krompir; biljni virusi; Y virus krompira; zaraza