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Sensitivity of field tests, serological and molecular techniques for *Plum Pox Virus* detection in various tissues

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ABSTRACT

Sensitivity of field tests (AgriStrip and Immunochromato), DAS-ELISA, two step RT-PCR and real-time RT-PCR for *Plum pox virus* (PPV) detection was tested in various tissues of apricot, peach, plum and damson plum trees infected with isolates belonging to PPV-D, PPV-M or PPV-Rec, the three strains present in Slovenia. Flowers of apricot and plum in full bloom proved to be a very good source for detection of PPV. PPV could be detected with all tested techniques in symptomatic parts of leaves in May and with one exception even in the beginning of August, but it was not detected in asymptomatic leaves using field tests, DAS-ELISA and partly also molecular techniques. PPV was detected only in some of the samples of asymptomatic parts of the leaves with symptoms and of stalks by field tests and DAS-ELISA. Infections were not detected in buds in August using field tests or DAS-ELISA. Field tests are useful for confirmation of the PPV infection in symptomatic leaves, but in tissues without symptoms DAS-ELISA should be combined or replaced by molecular techniques.

Key words: sharka, *Plum pox virus*, PPV, detection, field tests, DAS-ELISA, RT-PCR, real time RT-PCR

IZVLEČEK

OBČUTLJIVOST HITRIH TESTOV, SEROLOŠKIH IN MOLEKULARNIH TEHNIK ZA DETEKCIJO VIRUSA ŠARKE V RAZLIČNIH TKIVIH

Občutljivost hitrih testov (AgriStrip in Immunochromato), DAS-ELISA, dvostopenjske RT-PCR in RT-PCR v realnem času za detekcijo virusa šarke (*Plum pox virus*, PPV) smo proučevali v različnih tkivih dreves marelice, breskve, slive in cibore, okuženih z izolati PPV-D, PPV-M ali PPV-Rec. Ti trije različki so potrjeno navzoči v Sloveniji. Vzorci cvetov marelice in slive, odvzeti v času polnega cvetenja, so bili zelo primerni za detekcijo PPV. V delih listov z znaki okužbe je bila detekcija uspešna z vsemi tehnikami v maju in z eno izjemo tudi v avgustu. S hitrimi testi, DAS-ELISA in delno tudi z molekularnimi tehnikami nismo uspeli detektirati virusa šarke v listih brez znakov okužbe. S hitrimi testi in DAS-ELISA smo navzočnost PPV potrdili le v delu vzorcev iz asimptomatičnih delov listov z znaki okužbe in iz listnih pecljev ter v nobenem vzorcu brstov v avgustu. Hitri testi so torej primerni le za potrditev okužbe s PPV v listih z znaki okužbe. Če znaki okužbe niso vidni, je potrebno DAS-ELISA kombinirati ali nadomestiti z molekularnimi tehnikami.

Ključne besede: virus šarke, *Plum pox virus*, PPV, detekcija, hitri testi, DAS-ELISA, RT-PCR, RT-PCR v realnem času

1 INTRODUCTION

The introduction of infected plant propagation material is the most important mean of long distance spread of *Plum pox virus* (PPV). PPV is also transmitted by a number of aphid species and by vegetative propagation, including grafting. The length of incubation period is influenced by plant

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species, cultivar, time and mode of infection, vector species and virus strain. The data differ from some weeks to 8 years, but usually the incubation period takes 8 to10 months (Nemeth, 1986). Concentration of virus is low and symptoms are not visible in the early stage of infection. The expression of symptoms also differs considerably among cultivars. In a resistant plant the multiplication of the virus is limited and its spread slow (Polák, 2008), so the virus concentration is low. The concentration of virus varies also during the vegetation period and among tissues. Even in the same leaf there may be infected and virus-free zones (Nemeth, 1986). In the Mediterranean countries sampling of leaves is not recommended in summer months due to the high temperatures (EPPO, 2004) that cause low virus replication. Apart from mature leaves, flowers, small fruits as well as buds and bark in winter period are recommended for sampling by EPPO (EPPO, 2004).

Due to a possibility of low virus concentration, a great sensitivity and accuracy of detection technique is needed for successful and reliable detection. On the other hand cheap methods are desirable, since a lot of samples must be tested to ensure the sharka free status of planting material. Fast results are also needed, especially when testing imported planting material like graft-wood. The method must also be able to detect all the isolates. PPV has been classified into seven strains: PPV-M, PPV-D, PPV-Rec, PPV-EA, PPV-C, PPV-W, and PPV-T (Szathmáry and Palkovics, 2010), which differ in pathogenicity, symptom expression, host range, aphid transmissibility, and geographic distribution.

Sensitivity of different detection methods was therefore tested in different tissues of apricot, plum, damson plum and peach trees infected with isolates belonging to PPV-D, PPV-M or PPV-Rec, the three strains present in Slovenia. In particular suitability of field test for rapid detection was evaluated.

2 MATERIALS AND METHODS

2.1 Plant material

Plant material was collected from the same trees on 3rd of April, 1st of May and 1st of August, 2011 in a small garden in Maribor, NE Slovenia. On 3rd of May, 2011 additional samples were taken in a garden in Ljubljana, central Slovenia. Sampled host plants, the expression of symptoms on the sampled trees at the time of sampling and the tissue types tested are presented in Tables 1-3. Based on data from previous tests it is known that all trees (with exception of the two resistant cultivars 'Jojo' nad 'Katinka') have been infected for at least five years. For samples of asymptomatic leaves parts of leaves near the petiole (stalk) were used since Myrta *et al*. (2003) detected PPV most frequently in this part. In symptomatic leaves parts with symptoms were sampled separately from parts without symptoms. The tissue used for testing was excised and divided in three sub-samples. Individual sub-samples were used for testing with Immunochromato field test (NIPPON GENE Co., Ltd., Japan), with AgriStrip (BIOREBA AG, Switzerland) and with DAS-ELISA (BIOREBA AG, Switzerland).

2.2 Methods

Testing with AgriStrip (BIOREBA AG, Switzerland) was performed upon producer's protocol (available on http://www.bioreba.com/). For testing with Immunochromato field test (NIPPON GENE Co., Ltd., Japan), the sample was placed in the supplied extraction bag. Extraction solution (supplied by the producer) was added in 1:20 (w/v) ratio. After grinding, 0.65 ml of the extract was transferred to the sample tube and the test strip was placed in the extract. The results were recorded after 15 minutes.

DAS-ELISA was performed essentially as recommended by the producer (BIOREBA AG, Switzerland). Absorbance was read at 405 nm in a Sunrise Remote Control Reader (TECAN Austria GmbH). Samples were considered positive when the mean absorbance value of a sample exceeded the threshold. The threshold was set as at least three times the mean absorbance value (OD) of healthy controls as recommended by producer (http://www.bioreba.ch/?idpage=6).

Total RNA was isolated from extracts prepared for DAS-ELISA using RNeasy Plant Mini Kit (Qiagen, CA). 250 µl of RLT buffer (Qiagen, Germany) with 1% β-mercaptoethanol was added to 200 µl of extract. The isolation was than performed according to the manufacturer's instructions.

For reverse transcription 3 µl of isolated total RNA was added to 22 µl of reaction mix containing 50 pmol of oligo d(T)-based primer, 5 µl 5X M-MLV RT Buffer (Promega, USA), 5 µl dNTP mix (10 mM), 200 U M-MLV Reverse Transcriptase (Promega) and 20 U RNasin (Promega). The reactions were incubated for 10 minutes at 70 °C, placed on ice for 2 minutes and incubated further at 42 °C for 1 hour.

For the amplification, 47 µl of reaction mix consisting of 75 mM Tris–HCl at pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each of the primers and 2.5 U Taq DNA Recombinant Polymerase (Fermentas, UAB, Lithuania) were added to each tube containing 3 µl of the cDNA mix. The amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles with a thermal profile of 30 seconds at 94 °C, 30 seconds at 62 °C and 45 seconds at 72 °C and a final elongation step of 10 min at 72 °C. Primer pair P1/P2 (Wetzel *et al.*, 1991) was used for detection of PPV.

For sequencing (Macrogen, The Netherlands) unpurified DNAs obtained in the PCR with P1/P3M or P1/P3D (Wetzel *et al.*, 1991; Candresse *et al*., 1998) primer pairs were used. 3 µl of the cDNA mix was added to 47 µl of reaction mix containing 10 µl of 5X Colorless GoTaq Flexi Buffer (Promega), 3 µl of $MgCl_2$ (25 mM), 5 µl dNTP mix (10 mM), 20 pmol of each of the primers and 2.5 U of GoTaq Flexi DNA Polymerase. The amplification consisted of an initial denaturation step of 5 min at 94 °C, followed by 40 cycles with a thermal profile of 45 seconds at 94 °C, 30 seconds at 52 °C and 60 seconds at 72 °C and a final elongation step of 10 min at 72 °C.

The isolate type was determined by comparison of Slovenian sequences with sequences from the NCBI GenBank.

Real time RT-PCR was performed upon the protocol described by Mavrič Pleško *et al*. (2009). Ct values over 37 were considered negative.

3 RESULTS AND DISCUSSION

Plum 'Jojo' that possesses a highly reliable hypersensitive type of resistance (Neumüller and Hartmann, 2008) proved to be resistant also under high PPV infection pressure in Slovenia (Tables 1-3). Plum and apricot trees infected with PPV-M or PPV-D are growing in close vicinity and trees are infested with aphids. Since over 20 aphid species are known to be vectors of PPV the probability of transmission is very high. Nevertheless PPV could not be detected even with highly sensitive molecular methods in none of tested samples of this cultivar. Similar results were obtained with samples collected from plum 'Katinka' growing in the same garden. 'Katinka' is considered to be resistant by its breeders (Hartman, 1999), but has shown to be very susceptible to PPV-M by Kamenova and Milusheva (2005). In Maribor, PPV could be detected only in stalks using real time PCR (Table 2), but the Ct value was very low indicating a low concentration of the virus. Apricot 'Tyrinthos' showed a lot of symptoms on the majority of leaves, whereas apricot 'Boccuccia' proved to be much less susceptible with regard to symptoms on leaves. Few symptoms were observed on some leaves of 'Boccuccia' in May, but none in August. Symptoms were newer observed on leaves of plum rootstock. The scion part that has been showing clear PPV symptoms was cut down several years ago and the rootstock is growing as root suckers. On location Ljubljana symptoms were abundantly present on all sampled trees, which were infected with PPV-Rec, PPV-D or PPV-M.

Our results indicate that flowers in full bloom are a good tissue source for detection of PPV. Flowers in full bloom of apricot 'Tyrinthos' and 'Boccuccia', of unknown plum cultivar and of plum rootstock were suitable for detection of PPV, since the infection could be detected with all tested

techniques (Table 1). Negative results were obtained using field test for small closed flowers that gave positive result using DAS-ELISA and molecular techniques. Successful detection of PPV in flowers is in accordance with findings of Adams (1978) who could detect PPV using ELISA in flowers of all three tested varieties of plum. On the other hand Dosba *et al.* (1986) considered detection of PPV by ELISA in apricot and peach flowers as unreliable. Further tests need to be done. Young leaves taken from a rootstock during flowering had a lower concentration (estimated from the Ct value) of virus than flowers (Table 1). Infection could not be detected with field tests. Field tests also failed to detect infection in very small leaves of apricot 'Boccuccia' while DAS-ELISA showed suspiciously elevated OD values. Infection was confirmed using molecular techniques.

Table 1: Results of testing the samples collected on 3rd of April, 2011; Maribor, Slovenia.
Preglednica 1: Rezultati testiranja vzorcev zbranih 3. aprila 2011; Maribor, Slovenija.

Species, cultivar	Isolate	Tested	AgriStrip	Immuno chromato	ELISA (OD values)	RT-PCR	Real time (Ct)
apricot, 'Boccuccia'	D	very small leaves flowers in full bloom	neg. pos.	neg. pos.	susp.(0.092) pos. (0.771)	pos. pos.	pos. (30) pos. (19)
apricot, 'Tyrinthos'	M	flowers in full bloom	pos.	pos.	pos. (3.132)	pos.	pos. (15)
plum, unknown cultivar	M	flowers in full bloom	pos.	pos.	pos. (0.737)	pos.	pos. (21)
plum, 'Požegača' type	D	small closed flowers	neg.	neg.	pos. (0.334)	pos.	pos. (23)
plum rootstock	M	flowers in full bloom young leaves	pos. neg.	pos. neg.	pos. (0.682) pos. (0.134)	pos. pos.	pos. (21) pos. (24)
plum, 'Jojo'		flowers in full bloom	neg.	neg.	neg. (0.048)	neg.	neg.
plum, 'Katinka'		flowers in balloon stage	neg.	neg.	neg. (0.048)	neg.	neg.
		negative control			0.037		

pos. = PPV detected = potrjena okužba s PPV
neg. = PPV not detected = okužba s PPV ni potrjena
susp. = OD suspiciously elevated, but below the threshold

The results show that PPV is not always present in asymptomatic leaves or the amount of virus is very low therefore detection in latently infected trees is not always reliable. Detection of PPV with field tests and DAS-ELISA in mature leaves from infected trees depended much on the presence of symptoms (Tables 2 and 3). In our experiments PPV could be detected with all tested techniques in symptomatic parts of the leaves, even in the beginning of August when the temperatures were high. The only exception was Immunocromato test of symptomatic parts of the leaves taken from apricot 'Tyrinthos' in August. PPV infection was not detected in any of the tested samples from leaves without symptoms using field tests and DAS-ELISA in May and August. These results confirm the findings of several authors (Adams, 1978; Hamdorf, 1982; Myrta *et al.*, 2003), who described ELISA as unreliable when asymptomatic leaves were used. Using RT-PCR the presence of PPV in asymptomatic leaves was confirmed in 4 out of 9 samples from non-resistant trees (i.e. all trees except of 'Jojo' and 'Katinka'). Real time PCR gave somewhat better results. Nevertheless the virus could not be detected in 3 samples of asymptomatic leaves of non-resistant cultivars.

Table 2: Results of testing the samples collected on 1st and 3rd of May, 2011; Maribor and Ljubljana, Slovenia.
Preglednica 2: Rezultati testiranja vzorcev zbranih 1. in 3. maja 2011; Maribor in Ljubljana, Slovenija
Lw = leaves without symptoms = listi brez znakov
La = parts of the symptomatic leaves without symptoms
= asimptomatični deli listov z znaki
Ls = symptomatic parts of the leaves = simptomatični deli listov
Sw = stalks of the leaves without symptoms = peclji listov brez znakov
Ss = stalks of the symptomatic leaves = peclji listov z znaki
Svs = very small stalks = zelo majhni listni peclji

Location	Species, cultivar	Isolate	Symptoms	Tested	AgriStrip	Immuno chromato	ELISA (OD)	RT-PCR	Real time (Ct)
Maribor NE Slovenia	apricot, 'Boccuccia'	D	few symptoms on some leaves	Lw	neg.	neg.	neg. (0.025)	pos.	pos. (25)
				Sw	neg.	neg.	neg. (0.035)	pos.	pos. (24)
				La	pos.	neg.	neg. (0.023)	pos.	pos. (21)
				Ls	pos.	pos.	pos. (0.240)	pos.	pos. (17)
				Ss	pos.	neg.	neg. (0.028)	pos.	pos. (20)
	apricot, 'Tyrinthos'	M	a lot of symptoms on the majority of leaves	Lw	neg.	neg.	neg. (0.022)	neg.	neg. (37)
				Sw	neg.	neg.	neg. (0.027)	pos.	pos. (27)
				La	pos.	neg.	pos. (0.100)	pos.	pos. (18)
				Ls	pos.	pos.	pos. (0.386)	pos.	pos. (14)
				Ss	neg.	neg.	pos. (0.093)	pos.	pos. (21)
	plum, unknown cultivar	M	a lot of symptoms on the majority of leaves	Lw	neg.	neg.	neg. (0.023)	neg.	pos. (26)
				Sw	pos.	weak	pos. (0.110)	pos.	pos. (22)
				La	neg.	neg.	neg. (0.024)	neg.	neg.
				Ls	pos.	pos.	pos. (0.588)	pos.	pos. (13)
				Ss	pos.	weak	pos. (0.671)	pos.	pos. (18)
	plum, 'Požegača ' type	D	a lot of symptoms on the majority of leaves	Lw	neg.	neg.	neg. (0.025)	neg.	pos. (34)
				Sw	pos.	pos.	pos. (0.120)	pos.	pos. (21)
				La	weak	weak	susp. (0.057)	pos.	pos. (21)
				Ls	pos.	pos.	pos. (0.792)	pos.	pos. (13)
				Ss	pos.	weak	pos. (0.121)	pos.	pos. (19)
	plum rootstock	M	no symptoms	Lw	neg.	neg.	neg. (0.026)	neg.	neg. (38)
				Svs	n.t.	n.t.	n.t.	n.t.	n.t.
	plum, 'Jojo'		no symptoms	Lw	neg.	neg.	neg. (0.023)	neg.	neg. (37)
				Sw	neg.	neg.	neg. (0.023)	neg.	neg. (37)
	plum, 'Katinka'		no symptoms	Lw	neg.	neg.	neg. (0.023)	neg.	neg.
				Sw	neg.	neg.	neg. (0.024)	neg.	pos. (33)
Ljubljana, central Slovenia	apricot, unknown cultivar	D	a lot of symptoms on all leaves	La	pos.	neg.	pos. (0.111)	pos.	pos. (25)
				Ls	pos.	pos.	pos. (2.061)	pos.	pos. (18)
				Ss	pos.	pos.	pos. (0.408)	pos.	pos. (24)
	plum, unknown cultivar	Rec	a lot of symptoms on all leaves	La	pos.	neg.	pos. (0.206)	pos.	pos. (22)
				Ls	pos.	pos.	pos. (0.607)	pos.	pos. (18)
				Ss	pos.	pos.	pos. (0.369)	pos.	pos. (24)
	P. insititia = damson plum	Rec	a lot of symptoms on all leaves	La	neg.	neg.	pos. (0.274)	pos.	pos. (23)
				Ls	pos.	pos.	pos. (1.461)	pos.	pos. (16)
				Ss	pos.	pos.	pos. (0.242)	pos.	pos. (23)
	peach, unknown cultivar	M	symptoms on the majority of leaves	La	weak	neg.	pos. (0.305)	pos.	pos. (23)
				Ls	pos.	pos.	pos. (0.983)	pos.	pos. (18)
				Ss	neg.	neg.	susp. (0.073)	pos.	pos. (25)
	negative controls						0.021 – 0.028		

n.t. = not tested = ni testirano
pos. = PPV detected = potrjena okužba s PPV
neg. = PPV not detected = okužba s PPV ni potrjena
susp. = OD suspiciously elevated, but below the threshold

Table 3: Results of testing the samples collected on 1st of August, 2011; Maribor, Slovenia.
Preglednica 3: Rezultati testiranja vzorcev zbranih 1. avgusta 2011; Maribor, Slovenija.
Lw = leaves without symptoms = listi brez znakov
B = buds = brsti
La = parts of the symptomatic leaves without symptoms = asimptomatični deli listov z znaki
Ls = symptomatic parts of the leaves = simptomatični deli listov

Species, cultivar	Isolate	Symptoms	Tested	AgriStrip	Immuno chromato	ELISA (OD values)	RT-PCR	Real time Ct
apricot, 'Boccuccia'	D	no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.049) neg. (0.048	pos. pos.	pos. (26) pos. (30)
apricot, 'Tyrinthos'	M	a lot of symptoms	Lw La Ls B	neg. neg. pos. neg.	neg. neg. neg. neg.	neg. (0.031) neg. (0.037) pos. (0.254) neg. (0.043)	pos. pos. pos. pos.	pos. (25) pos. (26) pos. (21) pos. (27)
plum, unknown cultivar	M	a lot of symptoms	La Ls B	neg. pos. neg.	neg. pos. neg.	susp. (0.087) pos. (1.684) susp. (0.069)	pos. pos. pos.	pos. (22) pos. (16) pos. (24)
plum, 'Požegača'type	D	a lot of symptoms	Lw La Ls B	neg. neg. pos. neg.	neg. neg. pos. neg.	neg. (0.027) susp. (0.069) pos. (1.072) neg. (0.048)	neg. pos. pos. pos.	neg. (38) pos. (21) pos. (15) pos. (24)
plum rootstock	M	no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.048) neg. (0.046)	pos. pos.	pos. (28) pos. (22)
plum 'Jojo'		no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.034) neg. (0.026)	neg. neg.	neg. neg.
plum, 'Katinka'		no symptoms	Lw B	neg. neg.	neg. neg.	neg. (0.029) neg. (0.037)	neg. neg.	neg. neg.
			negative control			0.026 - 0.036		

pos. = PPV detected = potrjena okužba s PPV
neg. = PPV not detected = okužba s PPV ni potrjena
susp. = OD suspiciously elevated, but below the threshold

Using Immunochromato test infection could not be detected in asymptomatic parts of leaves with symptoms (Tables 2 and 3). AgriStrip and DAS-ELISA testing proved to more successful in May, but failed in August. In one sample tested in May PPV was not detected using even more sensitive molecular techniques. The observation that there may be infected and virus-free zones even in the same leaf (Nemeth, 1986) seams to hold also after using much more sensitive molecular techniques. Adams (1978) found that PPV was frequently undetected by ELISA test in asymptomatic parts of symptomatic plum leaves. The same was found for leaves of apricot 'Tyrinthos' by Myrta *et al.* (2003).

The use of leaf stalks is recommended by the producer of Immunochromato tests. Stalks were tested in May (Table 2). In contrast to leave blades without symptoms stalks taken from the same leaves occasionally gave positive results also with field test and DAS-ELISA. PPV could be confirmed in only some of the stalk samples from leaves with symptoms using field test and DAS-ELISA. Testing leaf stalks using molecular techniques in May proved to be more reliable, since infection was always confirmed in stalks of leaves with symptoms and in stalks of leaves without symptoms. The concentration estimated from Ct values of real time RT-PCR was always significantly lower in stalks from symptomatic leaves when compared with symptomatic parts of the leaves of the same sample. In contrast, the estimated concentration of PPV in stalks of the leaves without symptoms was always higher in comparison with the asymptomatic leave blades.

Our results show that buds are suitable for testing graft-wood in summer if molecular techniques are used. In Slovenia, grafting of *Prunus* is mostly

done in August therefore reliable detection in buds of graft-wood material is very important. Buds were tested only on one location. Infection was not confirmed by field test or by DAS-ELISA (Table 3). Some of the samples gave suspiciously elevated OD values using DAS-ELISA, but the infection needed to be confirmed with molecular techniques. Both tested molecular techniques confirmed the infection in all samples taken from non-resistant plants.

4 CONCLUSIONS

Sensitivity of different detection methods (field tests, DAS-ELISA, two-step RT-PCR and real-time RT-PCR) was tested in different tissues of apricot, plum, damson plum and peach trees infected with isolates of *Plum pox virus* PPV-D, PPV-M or PPV-Rec. Flowers of apricots and plums in full bloom proved to be a very good source for detection of PPV, since infection could be detected with all tested techniques. Detection in mature leaves depended on the presence of symptoms. PPV could be detected with all tested techniques in symptomatic parts of the leaves in May and with one exception even in the beginning of August. PPV was not detected in asymptomatic leaves and even in asymptomatic parts of the symptomatic leaves using field tests, DAS-ELISA and partly also molecular techniques. These results show that PPV is not always present in asymptomatic leaves or the amount of virus is very low; therefore, detection in latently infected trees is not always reliable. Additionally, the observation that there may be infected and virus-free zones even in the same leaf seams to hold also after using much more sensitive molecular techniques. Stalks were tested only in May and proved to be a good tissue source for detection with molecular techniques, since the presence of PPV was always confirmed in stalks from symptomatic as well as asymptomatic leaves. Reliable detection in buds is very important for testing of graft-wood. Unfortunately, infection could not be confirmed in buds in August using field tests or DAS-ELISA, therefore molecular techniques must be used for detection of PPV in graft-wood taken in summer.

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