

EVALUATION OF PRESENCE AND CONCENTRATION OF PPV IN ROOTSTOCKS DERIVED FROM *PRUNUS DAVIDIANA* (CARR.) FRANCH

Petra Pavelková¹, Tomáš Kiss¹, Tomáš Nečas¹

¹Department of Fruit Growing, Faculty of Horticulture, Mendel University in Brno, Valtická 337, 691 44 Lednice, Czech Republic

To link to this article: <https://doi.org/10.11118/actaun201967010121>

Received: 25. 7. 2018, Accepted: 16. 10. 2018

To cite this article: PAVELKOVÁ PETRA, KISS TOMÁŠ, NEČAS TOMÁŠ. 2019. Evaluation of Presence and Concentration of PPV in Rootstocks Derived from *Prunus davidiana* (Carr.) Franch. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 67(1): 121–131.

Abstract

Evaluation of the presence and concentration of PPV (Plum pox virus) in selected rootstocks was carried out in 2016–2017. For the purpose of the experiment we used rootstocks derived from crossbreeding of *Prunus davidiana* (Carr.) Franch, such as Cadaman and Barrier, and also a *P. davidiana* seedling. Peach seedling rootstock GF-305 was used as a control. The rootstocks were inoculated artificially with PPV strain M (Marcus). Both the rootstock and the inoculum were tested for presence of the virus by a serological method – semiquantitative DAS-ELISA test and molecular methods – RT-PCR, real-time RT-PCR and RT-LAMP. During the growing season the plants were evaluated for symptom intensity by using a scoring scale. The results show interdependency between symptom intensity and the amount of PPV in leaves, with DAS-ELISA test giving less positive samples than RT-PCR. The RT-LAMP and real-time RT-PCR methods were capable of revealing low concentrations of the virus even in symptom-free plants. The lowest PPV concentrations of all the four rootstocks were detected by real-time RT-PCR in *P. davidiana*. The highest PPV concentrations were detected in Barrier rootstock. In inocula, the lowest concentration was found in the inocula on Cadaman rootstock, whereas the highest PPV concentration was detected in the inocula inoculated on Barrier rootstock.

Keywords: sharka, stone fruit, symptoms, David's peach, DAS-ELISA, RT-PCR, RT-LAMP, real-time RT-PCR

INTRODUCTION

The Plum pox virus (PPV) belongs to the largest and most harmful group of ssRNA plant viruses, the family *Potyviridae*, genus *Potyvirus*. The group consists of more than 400 species, which makes

up one half of all the known plant viruses (Riechmann *et al.*, 1991; Polák *et al.*, 2010). For fruit growers this is a severe disease, infecting mainly plum, apricot and peach trees.

PPV was first identified in peach trees in Hungary in 1963 (Nemeth, 1965). In the Czech Republic,

its symptoms were first noticed in the eastern part of the country in the beginning of 1940 and since then the disease has spread to all regions of the country (Navrátil, 2006). Based on analyses, the PPV isolates are divided into seven strains with different biological, molecular and serological characteristics. The strains are: PPV-M, PPV-D, PPV-Rec, PPV-C, PPV-EA, PPV-W and PPV-T (Navrátil, 2006; Polák *et al.*, 2010). The Czech Republic suffers mainly from the PPV-D (Dideron) strain, which is less pathogenic, yet has been the infecting strain for more than 95 % of the infected trees in the country (Polák and Komínek, 2009). The highly pathogenic PPV-M strain is very rare in the Czech Republic (Polák *et al.*, 2010).

Severity of the disease hence depends on the virus strain as well as on susceptibility of the host species. There are differences in display of the symptoms, such as their intensity. Also the symptom onset time differs for the individual symptoms (Guillet-Bellanger and Audergon, 2006). In peach trees, the symptoms occur in leaves, fruits and blossoms. The leaves bear light green to greenish yellow stripes surrounding the venation or form various patterns, yet, the most frequently oak leaf mosaic. Sometimes epinasty and thickening of the leaf blade occur in the oldest leaves. The symptoms are more apparent in spring and usually fade away during summer months. Rings, patches or marbled patterns form on infected fruits. Sometimes the fruits are malformed. In some peach species, the symptoms also appear on petals as a clear discoloration. The intensity of symptoms may also vary significantly from year to year (Hluchý *et al.*, 1997; Llácer and Cambra, 2006; Salava and Polák, 2014).

PPV presence is usually confirmed using an immunoenzymatic test – the double-antibody sandwich ELISA (DAS-ELISA) (Clark and Adams, 1977). The same method can also be used for semiquantitative determination of a relative concentration of the virus in leaves (Paprštein, 2004). Yet, more frequently the PPV presence is being determined by molecular methods, by virus RNA detection using RT-PCR (Wetzel *et al.*, 1991; Levy and Hadidi, 1994) and real-time RT-PCR, which allows also quantification of the pathogen in the target tissues. Higher sensitivity of the RT-PCR and real-time RT-PCR than of DAS-ELISA make these methods more suitable for detection even of low concentrations of PPV in plant tissues (Rozák and Gálová, 2016). PPV can also be detected by RT-LAMP (reverse transcription loop – mediated isothermal amplification), which is a fast and sensitive diagnostic method (Varga and James, 2006).

Short time protective measures against the PPV include removal of the infected trees and cultivation of certified PPV-free planting material. Chemical protection against insect vectors is inefficient due to non-persistent transmission of PPV. Thus, the only efficient solution is to breed new resistant cultivars (Salava and Polák, 2014). Searching for new sources of PPV resistance and breeding new resistant cultivars of the *Prunus* species are the two most important goals of European breeding programs (Rubio *et al.*, 2003). At present, there are no suitable sources of PPV resistance for peach trees. A solution may be found in using wild *Prunus* species resistant to PPV that are close relatives to peach trees, for example an almond tree (*Prunus dulcis* (Mill.) D. A. Webb, syn. *Prunus amygdalus* Batsch) (Dicenta *et al.*, 2002; Pascal *et al.*, 2002; Rubio *et al.*, 2003.) and *Prunus davidiana* (Carr.) Franch (EPPO 1974).

The aim of this experiment was to verify the effect of *P. davidiana* and its derived rootstocks on the spread of PPV virus in experimental peach trees, and to confirm or refute their resistance against PPV and its detection by selected methods.

MATERIALS AND METHODS

The plant material used in the experiment was *P. davidiana* seedlings and vegetatively propagated Cadaman® (Avimag) and Barrier® rootstocks derived from the crossbreeding of *P. davidiana* with *Prunus persica* (L.) Batsch. Rooted cuttings of the rootstocks were obtained from an Italian company Vitrotree By Battistini (I). Highly sensitive peach seedling GF-305 rootstock was used as a control. Certified seeds were delivered by Pépinières Lafond (F).

The plants were cultivated in containers with a mixture of topsoil and a TS3 (Klasmann-Deilmann GmbH, DE) substrate (20 % dark and 80 % light more decomposed peat with pH 5.5–6.5; fertilizer: 1.0 kg/m³) with a slowly dissolving osmocote fertilizer. At the beginning of the experiment the individual variants constituted of 50 plants per variant.

All the plants were placed under the insect-proof net. The inoculation with PPV-M strain was done in august 2015 by chip-budding of buds from two different infected peach trees (cv. Symphonie and Cresthaven).

1. Evaluation of the PPV symptoms

Intensity of the symptoms was evaluated 5 times during the growing season in 2016 and 2017.

The plants were evaluated visually every 3 weeks since appearance of the first symptoms in May through to August. The evaluation was carried out using an intensity scale (0–4) according to Salava and Polák (2014):

1. No symptoms.
2. Very weak discolouration of the leaf veins or small diffusion spots (1 to 2 symptomatic leaves in a shoot)
3. Minor diffusion spots along secondary veins or minor mosaic (the first 3 to 4 symptomatic leaves in a shoot)
4. Yellowing of veins or moderate degree of oak leaf mosaic (the first 5 to 6 symptomatic leaves in a shoot)
5. Strong yellowing of veins, strong yellowish green rings and patterns of the oak leaf mosaic or epinasty and thickening of the leaf blades (the first 7 or more symptomatic leaves in a shoot)

2. Sampling and preparation of samples

Leaf samples were collected (in both years 2016 and 2017) in the second half of August after the last symptom evaluation. Leaf samples were collected from each plant having enough leaves for analysis of both the rootstock and the inoculum. Frozen (at -70°C) leaf samples were homogenized by a pestle in a mortar. The same homogenates were then used for the semiquantitative ELISA test and for RNA isolation followed by an analysis by RT-PCR, real-time RT-PCR and RT-LAMP to obtain comparable results.

3. Semiquantitative double antibody sandwich ELISA (DAS-ELISA)

Composition and preparation of the buffers used for the DAS-ELISA method was done according to Salava and Polák (2014). Antibodies and conjugated antibodies were purchased from Bioreba (CH). Testing and evaluation of the results was done in accordance with the methodology recommended by Bioreba (Bioreba, 2017) and modified to suit our task.

The homogenized sample mixed with an extraction buffer (0.25 g homogenized sample + 2.25 ml buffer) was diluted in a ten fold dilution ratio from 10 to 10^{-5} . Then 180 μl from each dilution point was pipetted into the 96 well plate. The sample was considered positive when its absorbance was higher than 0.100 after subtraction of the blank. Titer of the virus was expressed as the last (highest) dilution of the sample, in which

the sample was still positive. The virus titer is directly proportional to the concentration of the coat protein, which means that the relative concentration of the coat protein is a reciprocal of the virus titer (Svoboda and Polák, 2010).

4. RNA isolation

For RNA isolation 100 mg of homogenized sample was used. RNA was isolated using the Spectrum Plant Total RNA Kit by Sigma Aldrich (USA). The isolation was performed according to the protocol, version B. Each sample was diluted in 50 μl of an elution buffer (component of the kit) and stored at -20°C .

5. PCR and real-time PCR

Reverse transcription

Reverse transcription was performed according to the protocol described in the work of Eichmeier, Baranek and Pidra (2010). Twenty microliters of cDNA obtained from each RNA sample was used in subsequent PCR reactions.

PCR (housekeeping gene)

Detection of malate dehydrogenase – MDH, an internal positive control, was performed in each sample with the use of primers H968 (5'-GCATCTGTGGTTCTTGCAGG-3', forward) and C1163 (5'-CCTTTGAGTCCACAAGCCAA-3', reverse). One reaction of a 20 μl volume consisted of 1X GoTaq Buffer (Promega, USA), 0.25 mM dNTP's (Promega, USA), 0.1 μM of each primer, 1.5 mM MgCl_2 (Promega, USA), 1 U GoTaq G2 polymerase (Promega, USA), 2 μl cDNA and sterile nuclease free H_2O . The resulting product had 196 bp. The cycling conditions were: $95^{\circ}\text{C}/2$ minutes for polymerase activation, followed by 40 cycles of: 95°C for 30 s, 54°C for 45 s and 72°C for 60 s and a final elongation at 72°C for 5 minutes. The PCR products were separated on a 2 % agarose gel and visualized by GelRed (Biotium) on a transilluminator. Only samples with positive reaction were used for further analyses.

PCR (PPV detection)

To detect PPV, primers according to Levy and Hadidi (1994) were used. The PCR reaction mixture was identical to MDH housekeeping gene PCR reaction, but used with PPV primers. PCR cycling conditions were as in Levy and Hadidi (1994). The PCR products were separated on a 2 %

agarose gel and visualized by GelRed (Biotium) on a transilluminator.

For the detection and quantification of PPV by real-time PCR, primers described in Eichmeier *et al.* (2016) were used. GoTaq qPCR Master Mix (Promega, USA) chemistry was used to perform the real-time PCR, however the reaction volume, concentration of primers and amount of cDNA was the same as in Eichmeier *et al.* (2016). The cycling conditions were: 95 °C/2 minutes for polymerase activation, followed by 40 cycles of: 95 °C for 15 s and 60 °C for 60 s and by an analysis of the product's melting temperature. The sample was evaluated as positive, if its fluorescence curve crossed the threshold and the product's melting temperature was between 79 °C and 79.6 °C. The real-time PCR was performed on ECO real-time PCR cycler (Illumina, USA)

For PPV detection by PCR and real-time PCR each sample and control was tested in duplicates.

6. RT-LAMP

RT-LAMP was used for PPV detection in samples negative by DAS-ELISA method. Commercial bKIT PPV kit (Hyris, UK) optimized for real-time PCR cyclers was used. Reaction preparation and cycling conditions were performed according to the manufacturer (Hyris, UK). The kit is capable of detecting three different PPV strains: PPV-D, PPV-M and PPV-EA (Hyris, 2017). Each sample was tested in duplicate. RNA was the input material, as the kit is equipped with reverse transcriptase from the manufacturer. The RT-LAMP was performed in ECO real-time PCR cycler (Illumina, USA). The sample was evaluated as positive, if its curve of fluorescence crossed the threshold and the product's melting temperature was between 83 °C and 84.5 °C.

7. Standard curve set up and relative comparison of PPV concentrations in the samples

The sample with the lowest ct value (threshold cycle) from the real-time RT-PCR detection, i.e. the highest PPV concentration in leaf tissues was selected from the set of all the samples. RNA of this sample was diluted in a 7 point ten fold serial dilution in negative plant RNA. Each serial dilution point as well as the original sample was then transcribed to cDNA in the above described way. The cDNA of the serial dilution was then used to create a standard curve using the real-time PCR protocol described above. The standard

curve was established in ECO Study program (Illumina, USA) where the slope (k) of the linear regression line between the ct values and a log value of relative DNA concentration was used to calculate the amplification efficiency (E), $E = 10(-1/k) - 1$, where the value of 1 equals 100 % amplification. The squared regression coefficient (R^2) was determined after linear regression. The standard curve was used to perform a relative comparison of PPV concentrations in all the tested samples. Number 1 represented the most diluted dilution point in the standard curve (diluted 1,000,000 times) – this means the sample with the lowest relative PPV concentrations in tissue. The undiluted sample of the standard curve was expressed as number 1,000,000 and represented a 1,000,000 times higher relative concentration than number 1. In this way, we compared the relative concentrations of PPV in the two parts of the plant – the rootstock and the inoculated infected part – for all the rootstock variants.

8. Statistical evaluation

Tukey HSD test and analysis of variance were used for evaluation of the results of individual rootstocks and of the inoculum and rootstock parts separately. The goal was to evaluate differences in symptom manifestation and relative PPV concentrations between inoculum and rootstock parts as well as between rootstock types. Furthermore, correlation between relative PPV concentration (DAS-ELISA and real-time PCR) and the symptom intensity was evaluated. All the statistical analyses were calculated in Statistica 12 software.

RESULTS

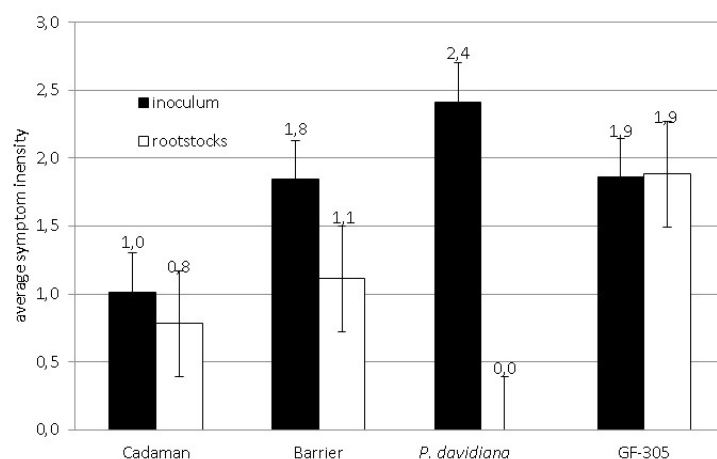
The average values of relative virus concentration and symptom intensity of all the tested rootstocks are shown in Figs. 1, 2 and 4. In leaves, the symptoms were usually apparent as vein discolourations and as a mosaic pattern located around the leaf veins. Leaves of inocula usually exhibited stronger symptoms than leaves of the rootstocks. Besides the inocula leaves had clear signs of blade thickening. *P. davidiana* did not exhibit PPV symptoms on its leaves however, the inoculum on this rootstock exhibited the strongest symptoms among all inocula and rootstocks (Fig. 1). In the remaining rootstocks, the symptoms occurred in various intensities (0–4 of the evaluation scale) and were found both on the rootstock and inoculum leaves. Among rootstocks, the strongest PPV symptoms were observed on control rootstock

GF-305, which also exhibited strong leaf blade deformations.

In plants with the strongest PPV symptoms on leaves the DAS-ELISA titers were within the range of 10^3 to 10^4 ; in symptom-free plants the titer ranged up to 10^2 at the highest, however, symptom-free *P. davidiana* rootstocks were negative in DAS-ELISA (Fig. 2 and Tab. I). The highest relative PPV concentration was detected in inoculum leaves

on the control rootstock GF-305 and the second highest in the leaves of inoculum on *P. davidiana* rootstock. The highest relative PPV concentration in rootstock leaves was detected in Barrier rootstock and the second highest in the leaves of the control rootstock GF-305.

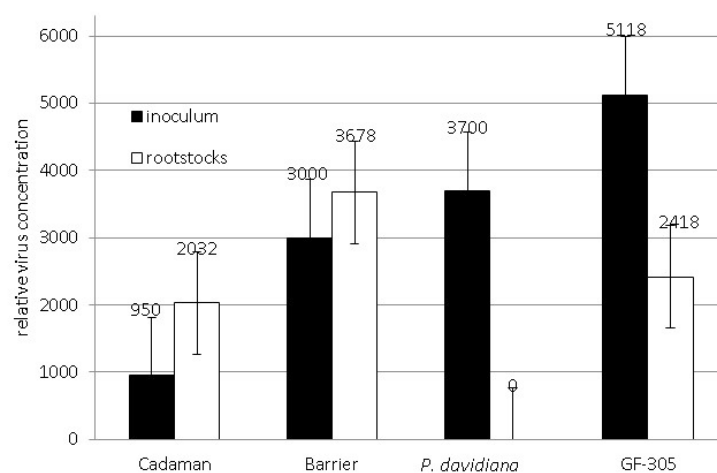
To perform the comparison of the relative PPV concentration in all the samples by real-time RT-PCR, the sample with the lowest ct value of



1: Average symptoms intensity calculated from five evaluations within the growing season.

I: Average relative PPV concentrations determined based on the titer of the individual variants using semiquantitative DAS-ELISA test.

rootstocks	ELISA inoculum	ELISA rootstock
Cadaman	9.50×10^2	2.03×10^3
Barrier	3.00×10^3	3.68×10^3
<i>P. davidiana</i>	3.70×10^3	0.00
GF-305	5.12×10^3	2.42×10^3



2: Average relative PPV concentrations determined based on the titer of the individual variants using semiquantitative DAS-ELISA test.

15.41 was used to establish a standard curve (Fig. 3, Tab. II). The efficiency of the real-time RT-PCR of set up standard curve was 101.46 % with the slope value of -3.29 (Tab. 2), which was close to the ideal value of -3.32 . The ct values of the standard curve ranged from 15.74 to 35.99, which covered most of the ct value range of the tested samples (15.41

to 35.94). The relative PPV concentration values in the samples ranged from 0.8 up to 1 347 712.9.

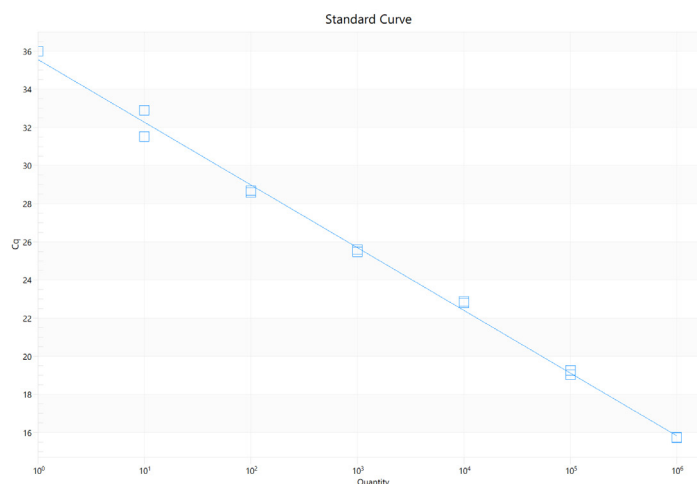
The highest relative PPV concentration measured by real-time RT-PCR was detected in Barrier rootstock and in the inoculum of this rootstock (Fig. 4), showing 1.2 times higher relative PPV concentration in the rootstock part

II: Ct values of the serial dilution and standard curve characteristics of the sample with ct value 15.41 used for comparison of relative PPV concentration in tested samples by real-time RT-PCR.

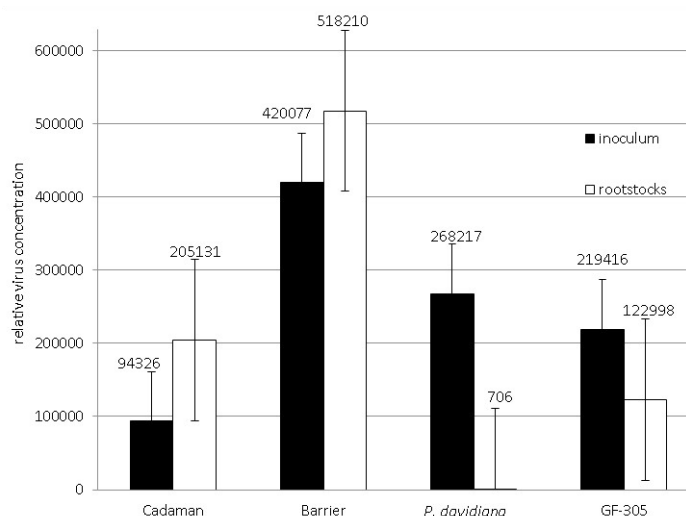
ct sample	Efficiency %	Slope	R2	ct value						
				Dilution						
				Undiluted sample	10x	100x	1,000x	10,000x	100,000x	1,000,000x
15.41	101.46	-3.29	0.99	15.74	19.15	22.84	25.53	28.64	32.21	35.99*

ND – not detected

* – only one of the duplicates was positive



3: A standard curve for the sample with ct 15.41 in the range of 10^0 – 10^6 . Characteristics: E: 101.46 %, k: -3.29 , R2: 0.99.



4: Average relative PPV concentrations measured in the individual variants using real-time PCR.

than in the inoculum. The second highest PPV concentration was detected in the inoculum of *P. davidiana* rootstocks. The lowest amount of PPV was detected in the *P. davidiana* rootstock, where relative PPV concentration in the rootstock part was about 380 times lower than in its inoculum. In Cadaman rootstock, the relative PPV concentration was about 2.2 times higher than in its inoculum. Surprisingly, in the control rootstock GF-305, 1.8 times higher relative PPV concentration was detected in the inoculum than in the rootstock.

RT-LAMP method was used for PPV detection in samples negative by DAS-ELISA, however, positive by real-time RT-PCR, i.e. samples with lower PPV concentration. RT-LAMP method confirmed the results obtained by real-time RT-PCR.

The detection efficiency of DAS-ELISA method was 64 %, where from total of 133 samples only 85 samples were tested positive (Tab. III). With real-time RT-PCR, on the other hand, the PPV was detected in all 133 samples, showing 100 % detection efficiency. This confirms that all the evaluated plants were inoculated with PPV successfully. PPV detection by RT-PCR failed to detect 5 samples which were positive by real-time RT-PCR and detection by RT-LAMP failed to detect only two samples positive by real-time RT-PCR. Results show that the detection efficiency of both methods, the RT-PCR and RT-LAMP, is relatively similar (96.2 and 96.0 %, respectively).

The Tukey's HSD test revealed statistically significant differences in symptom intensity in the inocula of the individual rootstocks, namely, between the Barrier and Cadaman rootstocks and between the Cadaman and *P. davidiana* rootstocks.

The comparison of relative PPV concentrations measured by semiquantitative DAS-ELISA revealed statistically significant differences between the inocula on Barrier and GF-305 rootstocks and between Cadaman and GF-305 rootstocks. The real-time RT-PCR also revealed statistically significant differences between the relative PPV concentrations of inocula of Cadaman and GF-305 rootstocks. (Tab. IV)

For the rootstocks themselves, the Tukey's HSD test revealed statistically significant differences only in the symptom intensities (Tab. V). The differences were observed between the Cadaman and GF-305 rootstocks, between the *P. davidiana* and GF-305 rootstocks and between the Barrier and *P. davidiana* rootstocks.

Relative PPV concentrations in the rootstocks themselves showed no statistically significant differences. (Tab. V.)

Next, correlations between the symptom intensity and the relative PPV concentration in leaves measured by DAS-ELISA and real-time RT-PCR was studied.

The strongest correlation among inocula was shown between the relative PPV concentration of DAS ELISA and real-time RT-PCR methods (0.68;

III: Numbers of PPV positive samples detected by the selected methods.

Method	Total samples	Detected	Rootstock	Inoculum	Efficiency
DAS-ELISA	133	85	39	46	64.0 %
RT-PCR	133	128	64	64	96.2 %
real-time RT-PCR	133	133	66	67	100 %
visual check	133	94	42	52	70.7 %
RT-LAMP	49**	47	26*	21*	96.0 %

**Only samples tested, which were not detected by DAS-ELISA as positive

*RT-LAMP total number of the tested rootstocks 28 samples; * RT-LAMP total number of the tested inoculum shoots 21 samples.

IV: Significance of the differences in symptom occurrence and PPV concentrations in the inocula detected using DAS-ELISA and real-time RT-PCR.

Inoculum	Symptoms average	Tukey's HSD test	DAS-ELISA average	Tukey's HSD test	Real-time RT-PCR average PPV	Tukey's HSD test
Barrier	2.08	a	1420.00	a	189945.00	ab
Cadaman	1.26	b	796.42	a	72400.60	a
<i>P. davidiana</i>	2.38	a	2033.33	ab	268217.00	ab
GF-305	1.82	ab	4578.95	b	196319.60	b

V: Significance of the differences in symptom occurrence and PPV concentrations in the tested rootstocks detected using DAS-ELISA and real-time RT-PCR.

Rootstock	Symptoms average	Tukey's HSD test	DAS-ELISA average	Tukey's HSD test	Real-time RT-PCR average PPV	Tukey's HSD test
Barrier	1.57	bc	3273.33	a	354422.20	a
Cadaman	1.01	ab	1909.62	a	174793.90	a
<i>P. davidiana</i>	0.00	a	0.00	a	355.00	a
GF-305	1.82	c	2163.16	a	116165.20	a

VI: Correlation coefficient values representing significant dependence between the symptom occurrence and the detected concentrations

INOCULUM								
Methods	Average	Correlation (P < 0.05)	Methods	Average	Correlation (P < 0.05)	Methods	Average	Correlation (P < 0.05)
DAS-ELISA	1856.40	0.68	DAS-ELISA	1786.92	0.21	real-time RT-PCR	128030.30	0.34
real-time RT-PCR	125130.90		symptoms	1.58		symptoms	1.60	
ROOTSTOCKS								
Methods	Average	Correlation (P < 0.05)	Methods	Average	Correlation (P < 0.05)	Methods	Average	Correlation (P < 0.05)
DAS-ELISA	2175.10	0.79	DAS-ELISA	2058.17	0.56	real-time RT-PCR	181526.60	0.41
real-time RT-PCR	181526.60		symptoms	1.19		symptoms	1.20	

Tab. IV). Weak correlation was shown between the relative PPV concentration of DAS-ELISA and the symptom intensity and the same applies to the correlation between relative PPV concentration of the real-time RT-PCR results and the symptom intensity.

At rootstocks the strongest correlation was again shown between the relative PPV concentration of DAS-ELISA and real-time RT-PCR results (0.79; Tab. VI). Between the symptom intensity and the relative PPV concentration of DAS-ELISA results in rootstocks moderate correlation was measured. However, between the symptom intensity and the relative PPV concentration of real-time PCR results weak correlation was shown.

DISCUSSION

Based on the results of this study, DAS-ELISA could not detect low titers of the virus. When compared with real-time RT-PCR the DAS-ELISA method was only capable of detecting samples with ct values within the range of 15.41 to 31.72, which means relative PPV concentrations from

10^4 to 10^1 according to the DAS-ELISA method. Ct 31.72 was measured in an undiluted sample with absorbance of 0.111, i.e. very closely above the limit determining a 100 % PPV positive sample. The real-time RT-PCR and RT-LAMP methods generally discovered the highest number of positive samples with low virus concentrations. With the exception of a few samples, both methods produced equal results. The high sensitivity of the real-time RT-PCR and RT-LAMP is also mentioned in the work of Varga and James (2006).

According to the results of symptom intensities of PPV infection on leaves and on subsequent verification of PPV presence and measurement of its relative concentration by semiquantitative DAS-ELISA, RT-PCR, real-time RT-PCR and RT-LAMP, the rootstocks were evaluated according to their response (resistance) to PPV. Ll acer *et al.* (2007) state that when evaluating resistance many factors must be taken into account: virus isolate, source of the inoculum, the rootstock, host genotype, inoculation method and the time of inoculation, physiological state of the host, evaluation time,

conditions inside the greenhouse, number of plants tested for each genotype and number of evaluated growing cycles.

The results of this experiment reflect these factors and it was proven that *P. davidiana* decreases considerably the amount of the virus in the plant's tissue. In the first year after inoculation, PPV was detected by DAS-ELISA only in the leaves of inoculum, while the *P. davidiana* rootstock remained negative. In the *P. davidiana* rootstock part itself the virus was only detected by RT-PCR, real-time RT-PCR and RT-LAMP methods. At the same time, symptoms were not observed in leaves of this rootstock, which agrees with the claims of numerous authors that in *P. davidiana* symptom masking may happen or the symptoms may be very weak (Decroocq *et al.*, 2005, Rubio *et al.*, 2010, Pascal *et al.*, 2002). Still, the inoculum infected with PPV inoculated on this rootstock exhibited very strong symptoms. (Fig. 1) However, in the interspecific hybrid Cadaman (*P. davidiana* x *P. persica*) no reduction of symptoms in leaves of the rootstock, nor the inoculum was observed. Yet, the concentration of the virus in tissues were lower than in Barrier rootstock, which exhibited higher symptom intensity and relative PPV concentration than Cadaman rootstock in both the inoculum and the rootstock itself. This might indicate that Cadaman remains genetically closer to *P. persica* than to *P. davidiana*.

Damsteegt *et al.* (2007) state that for positive peach plants with strong mosaic symptoms in leaves the average absorbance value in ELISA test was 1.5 to 2.5. Absorbance values for positive symptom-free plants were changing from 0.1 to 0.5 depending on the species (for negative controls the values were 0.0). The research presented here brought similar results – for the plants with the strongest symptoms in leaves the average absorbance value in DAS-ELISA test

was 2.438 and the titer ranged from 10^3 to 10^4 . For the symptom-free plants the average absorbance was 0.33 and titers 10^2 at the highest.

Pascal *et al.* (2002) observed that genotypes derived from *P. davidiana* showed PPV symptoms limited to one or two leaves only with the heterogenous spread of the symptoms regardless of the studied genotype. In our study, the symptom intensities observed in hybrids derived from *P. davidiana* were higher, where mostly 5–6 symptomatic leaves were observed on a shoot.

An extensive evaluation of a peach gene pool collection (germplasm) showed that PPV resistance is probably quantitatively based and is manifested as a decrease in symptom intensity (Cirilli *et al.*, 2017). For this reason, it is important to disclose the genetic architecture of the response of peach trees and their relative species to PPV infection, which is necessary for pyramiding of resistant genes and for development of more tolerant species and rootstocks. PASCAL *et al.* (2002) state that immunity to potyvirus was also found in two almond cultivars 'Ferragnès' and 'Ardéchoise' with a self-pollinating cultivar 'Lauranne'® (R 916) also exhibiting a very high level of resistance towards PPV-M. These results confirm that *P. amygdalus* is a potential source of resistance to pests and diseases of peach trees and that these species should be directly utilized in rootstock breeding programs. When selecting peach species, breeding programs will have to take into account characteristics of the fruit and in this context the *P. davidiana* has an advantage of its closer genetical relatedness to *P. persica* (Pascal *et al.*, 2002).

Labonne *et al.* (1989) claim that the detected high level of resistance against PPV-M in *P. davidiana* is comparable to the level observed in *P. amygdalus*, especially in the second phase of the growing season, when multiplication and spread of PPV in a *P. davidiana* was apparently null. The same can

also be expected under natural conditions, where the levels of virus transmission by aphid vectors are noticeably low (Labonne *et al.*, 1989).

CONCLUSION

Based on the results of symptom intensities of PPV infection on leaves and on subsequent confirmation of PPV presence and its relative concentration by semiquantitative DAS-ELISA, RT-PCR, real-time RT-PCR and RT-LAMP the rootstocks were evaluated according to their response (resistance) to PPV. The results showed that the *P. davidiana* rootstock itself lowers significantly the amount of the virus in plant's tissue, the virus could be detected by the DAS-ELISA only in the growing inoculum, whereas in the rootstock itself, which was symptom-free, the virus was only detected by molecular methods. Of all the four tested rootstocks it was the *P. davidiana* seedling, where the relative PPV concentration, measured by real-time RT-PCR, in the rootstock parts was found to be the lowest (706.0), however,

the relative PPV concentration in its inoculum (268 217) was the second highest among inocula. At Cadaman rootstock, which derived from *P. davidiana*, the rootstock itself (205 131) showed the second highest relative PPV concentration measured by real-time RT-PCR among all rootstocks, however relative PPV concentration in its inoculum (94 326) was on the other hand the lowest among inocula. The highest relative PPV concentration in both inoculum (420 077) and the rootstock itself (518 210) was in the Barrier rootstock. Finally, the control GF-305 rootstock showed in both the inoculum (219 416) and the rootstock itself (122 998) the second lowest relative PPV concentrations measured by real-time RT-PCR among inocula and rootstocks. Based on these results it is not possible to prove, but also refute the resistance of *P. davidiana* and its derived rootstock Cadaman against PPV as from one hand they lowered the amount of PPV in their tissues, but this effect was not observed in all parts of the plant, i.e. inoculum and the rootstock. Moreover in Barrier, which is also derived from *P. davidiana*, the effect of PPV reduction was not observed at all. More studies are needed for exploitation of the effect of *P. davidiana* and its derived rootstocks on the PPV infection.

Acknowledgement

This research was funded by NAZV project QJ1510081 by the Ministry of Agriculture of the Czech Republic and with a financial support of the Mendel university Internal grant agency (IGA) from project [SP5170911].

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Contact information

Petra Pavelková: xpavelk8@node.mendelu.cz
Tomáš Nečas: necast@zf.mendelu.cz