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Influence of D and Rec strains of plum pox virus on phenolic profile and antioxidant capacity of fresh plum fruits of 'Čačanska Lepotica' cultivar

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Abstract

Aim of study: To investigate the changes in chemical composition of fresh plum fruits cv. 'Čačanska Lepotica' prompted by the presence of various strains of plum pox virus (PPV).

Area of study: Serbia.

Material and methods: In an experimental orchard of 'Čačanska Lepotica' plum cultivar, fruits were picked from virus-free and PPV-infected trees (PPV-D and PPV-Rec strains) in four harvest stages in 2017 and 2018. Fruits were further analyzed on total phenolics, flavonoids, anthocyanins, antioxidant capacity and selected phenolics.

Main results: The results indicate that virus infection causes chemical changes to a certain extent, but mostly in initial harvest stages, while the values are equal in later stages. In the last harvest stage, as the most utilizable in commercial purposes, only chlorogenic acid content was affected in 2017, while in 2018 contents of neochlorogenic acid and chrysanthemin were altered by the PPV infection. Total contents of flavonoids and phenolics revealed no influence of virus infection during both 2017 and 2018, while PPV-Rec infected samples were richer in anthocyanins under heavy rainfall during summer months of 2018. Given the number of identified compounds (10) and the vast experimental data, it might be concluded that influence of PPV infection on chemical composition of 'Čačanska Lepotica' plum fruits was quite limited.

Research highlights: Plum cultivar 'Čačanska Lepotica' should be considered as highly tolerant cultivar to PPV, and can be grown in heavily infected environment with no risk. Therefore, it might be a great replacement for sensitive plum cultivars, such as 'Požegača' and 'Čačanska Rodna'.

Additional key words: Sharka; *Prunus domestica* L; harvest stages; anthocyanins; hydroxycinnamic acids; flavonols.

Abbreviation used: ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)); ACLSV (Apple chlorotic leafspot virus); ApMV (Apple mosaic virus); CE (catechin equivalents); C3GE (cyanidin-3-glucoside equivalents); DAS-ELISA (double-antibody sandwich ELISA); ELISA (enzyme-linked immunosorbent assay); FW (fresh weight); GAE (gallic acid equivalents); IC-RT-PCR (immunocapture reverse transcription polymerase chain reaction); MRLSV (Myrobalan latent ringspot virus); PDV (Prune dwarf virus); PNRSV (Prunus necrotic ringspot virus); PPV (Plum pox virus); PPV-Rec (PPV recombinant).

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Introduction

The world estimated production of plums (*Prunus domestica* L.) and sloes (*Prunus spinose* L.) for 2017 was about 11.7 million tons (http://www.fao.org/faostat/en/#data). One of the top producers was Serbia with the average annual production of plum fruits in the period 2010−2019 of 442.250 tons. Plum is a leading stone fruit species in Serbian agriculture by a virtue of favorable soil and climate conditions, economic interest and tradition. Variety structure is diverse, from autochthonous to newly bred cultivars. Plum pox virus (PPV) was first reported in 1932 on plums in Bulgaria and afterwards in Serbia (Josifović, 1937). PPV is the causal agent of the Sharka disease, that is considered as the most detrimental viral disease of stone fruits. It induces symptoms on leaves, flowers, fruits and seeds. In sensitive cultivars, symptoms on fruits may be severe making them unusable and unmarketable. Beside direct damage to the fruits, they may ripen earlier and drop prematurely.

PPV belongs to the genus *Potyvirus* in the *Potyviridae* family. Based on biological, serological and molecular characteristics, ten PPV strains have been recognized so far: PPV-M, -D, -EA, -C, -Rec, -W, -T, -CR, -An and -CV. PPV-M, -D and -Rec are most dispersed and they are considered as major strains. Other strains are of minor importance and they are geographically or host limited.

Plum species and cultivars differ in their susceptibility to PPV. Plum 'Požegača', old autochthonous Serbian cultivar which was a dominant plum cultivar in Serbia for decades, is very sensitive to PPV. Today, it is present only in old and abandoned orchards. PPV-infected 'Požegača' fruits are deformed and unusable for fresh consumption or for processing into brandy, prunes or jams. In contrast to sensitive, tolerant plum cultivars may be infected with PPV and express less or more severe Sharka symptoms on leaves. Fruits on these cultivars are normally developed and have no visible damages, or they occur very rarely in some years (Jevremović, 2013). Plum 'Čačanska Lepotica' was developed from the cross of 'Wangenheims Frühzwetsche' and 'Požegača' at Fruit Research Institute in Čačak, Serbia, and released in 1975. It is cultivated in many countries throughout Europe. Fruits of 'Čačanska Lepotica' are intended for fresh consumption, but also for the production of high-quality brandies.

Plums contain various types of phenolic phytochemicals, including flavonoids, phenolic acids, anthocyanins and tannins, contributing to the high antioxidant capacity of this fruit (Chun *et al*., 2003; Kim *et al*., 2003; Walkowiak-Tomczak, 2008). Due to its chemical composition, it is not surprising that fresh plum fruits taken in our daily diet possess copious health benefits on humans, mostly in preventing and surpassing various diseases (Igwe & Charlton, 2016). The content of polyphenolics in fruits, as secondary metabolites, highly depends of various parameters, such as cultivar specificities, degree of ripeness, agro-technical treatments, geographic origin, postharvest storage conditions, as well as virus presence (Miletić *et al*., 2012; Usenik *et al*., 2015; Sahamishirazi *et al*., 2017; Drkenda *et al*., 2019; Cabrera-Bañegil *et al*., 2020; Radović *et al*., 2020). Degree of chemical composition changes, prompted by virus infection, mostly depends on cultivar tolerance to certain virus. There are numerous published studies regarding the influence of various parameters on polyphenolic content of fresh plum fruits, but not that many references exist about the effect of virus presence on polyphenolic profile, especially during on-tree ripening. Usenik *et al*. (2015) investigated the quality of fresh plums during last three weeks of ripening, comparing uninfected, short-term infected and long-term infected trees. The study revealed that PPV infection altered the ripening process, and content of nutritive and bioactive compounds, drawing the conclusion about low tolerance of 'Domača češplja' plum cultivar to PPV. Sochor *et al*. (2015) revealed that virus presence in genetically modified plum cultivar 'Honeysweet' greatly influenced the total and individual polyphenolic contents, as well as antioxidant capacity, compared to the virus-free plum samples.

The main objective of this research was to evaluate the influence of PPV-D and PPV-Rec strains on chemical composition of fresh plum fruits of 'Čačanska Lepotica', a cultivar which is considered to be tolerant to PPV.

Material and methods

Experimental orchard

The trial was set up in an experimental plum 'Čačanska Lepotica' orchard planted in spring 2008 in the village Ostra, municipality Čačak, Serbia (43°54'54.56"N, 20°30'07.31"E). Orchard was initially set up to study the spread of PPV-D and PPV-Rec strains within the orchard from internal and external sources. The orchard was planted with 400 virus-free plum trees at 4 x 3.5 m spacing and maintained using integrated pest and disease management system.

Average monthly and yearly values of air temperature (°C) and precipitation (mm) for the studied years was recorded each month from the automatic weather station located in Mrčajevci (5 km from the orchard) (Table 1).

IC-RT-PCR analysis

From 2008, all trees within the orchard were annually visually checked on the presence of Sharka-like symptoms and then tested by ELISA for the PPV presence. All ELISA positive samples were further tested using IC-RT-PCR in order to determine the present strain. The sample of each tree consisted of 20−25 randomly collected leaf samples around the canopy of the tree. IC was performed using polyclonal antibodies (Fruit Research Institute, Čačak) and

Table 1. Average monthly and yearly values of air temperature and precipitation for the investigated period (2017–2018).

Month	Air temperature (°C)		Precipitation (mm)	
	2017	2018	2017	2018
April	10.3	16.5	89.4	34.4
May	15.9	17.9	90.2	35.8
June	21.6	20.2	38.4	97.6
July	22.5	21.1	46.8	150.4
August	22.5	22.9	42.0	53.6
September	16.6	17.4	52.4	28.6
October	10.7	13.2	79.2	14.4

RT with random hexamer primers using Maxima Reverse Transcriptase (Thermo Fisher Scientific, USA). Each sample was tested in four separate PCR reactions using PPV-D and PPV-M specific primers. For the amplification of 467 bp fragments located in the C-ter Nib–Nter-CP coding region primer pairs P4/P3M and P4/P3D were used (Candresse *et al*., 1998). To amplify 880 bp and 468 bp fragments located in the CI coding region, CIP-M/CIP-MR and CIP-D/CIP-DR primer sets were used, respectively (Kamenova *et al*., 2011). PCR reactions were carried out in TPersonal thermal cycler (Biometra, Germany) and amplified products were analyzed in 1.5% agarose gel. Gel was stained with ethidium-bromide and visualized with a Gel Doc EZ System (Biorad, USA) using UV tray.

The presence of the amplified fragment of the expected size with each primer pair was considered as positive reaction. Strain typing of the isolates was done according to the PCR results, as described earlier by Jevremović (2013) (Table S1 [suppl]). Based on the PPV strain-typing results, we selected five PPV-D, five PPV-Rec and five PPV-free trees for fruit analysis.

Selected trees were also tested on the presence of other viruses infecting stone fruits to exclude their possible influence on the examined traits. The analysis on the PDV, PNRSV, ACLSV, MRLSV and ApMV presence was done by DAS-ELISA. Analysis was done with the commercial reagents (BIOREBA AG, Switzerland, for PDV, PNRSV, ACLSV and ApMV; and BIORAD, France, for MLRSV) with recommended protocols by the producers.

Fruit sampling

After IC-RT-PCR analysis, fruit samples were collected from 15 selected trees: 5 PPV-D infected, 5 PPV-Rec infected and 5 PPV-free as a control. Over the seasons examined, fruits were hand-picked at four different harvest stages (HS1, HS2, HS3, HS4) at seven-day intervals, starting from a fully green fruits (HS1) and ending up with deep blue fully ripen fruits (HS4), as described in Table 2. Thirty fruits with no mechanical injuries were picked from the same selected trees at every harvest stage during both harvest years (2017, 2018). Fruits were picked from all parts around the canopy. During every harvest stage (15 trees × 30 plums), 450 plum fruits were collected, or 1,800 plum samples per harvest year (450 plums × 4 harvest stages).

Extraction and determination of total anthocyanins, flavonoids, phenolics and antioxidant capacity

From each plum tree, ten plum fruits out of thirty were randomly selected to make a single extract. Whole edible parts of plums were frozen into the liquid nitrogen, then grinded and homogenized using a stainless-steel blender, until the fine powder was obtained. Grinded plum samples (10 g) were mixed with 25 mL of 96% ethanol and ultrasonicated. After 30 min of extraction, the mixture was centrifuged in two sequential times for 15 min at 3500 rpm, and supernatant was filtered through a 0.45 mm Minisart filter before analysis. In total, 15 extractions were performed for every harvest stage: 5 extractions from plum fruits from PPV-free selected trees, 5 from plums from PPV-D infected and 5 from plums from PPV-Rec infected trees. Then, 5 extracts from the same infection origin were mixed into the one, resulting in 3 working extracts (PPV-free, PPV-D and PPV-Rec), for each harvest stage and each harvest year. The obtained extracts were used for the determination of flavonoid and polyphenolic contents, antioxidant capacity and HPLC analyses. The identical extraction procedure was repeated, but with 25 mL of 96% ethanol/HCl (85:15 v/v), in order to obtain extract for anthocyanin content. All these determinations were performed in triplicate, and results were presented as mean value of three measurements ± standard deviation.

The monomeric anthocyanin pigment content of the aqueous extracts was determined using the pH differential method previously described (Prior *et al*., 1998; Liu *et al*., 2002). The results were expressed as mg C3GE/100 g FW.

Table 2. Selected harvest stages and virus-presence indication for 'Čačanska Lepotica' plum fruits collection over two successive harvest years.

Harvest stage	2017			2018		
	Neg	PPV-D	PPV-Rec	Neg	PPV-D	PPV-Rec
1	17HS1-N [a]	17HS1-D	17HS1-R	18HS1-N	18HS1-D	18HS1-R
2	17HS2-N	17HS2-D	17HS2-R	18HS2-N	18HS2-D	18HS2-R
3	17HS3-N	17HS3-D	17HS3-R	18HS3-N	18HS3-D	18HS3-R
4	17HS4-N	17HS4-D	17HS4-R	18HS4-N	18HS4-D	18HS4-R

[a] Initial number indicates the harvest year (2017, 2018); HS abbreviates the harvest stage; the second number stands for serial number of harvest; N, D, R stand for negative sample (virus-free), sample infected with PPV-D strain and sample infected with PPV-Rec strain, respectively.

Total flavonoid content was determined by a colorimetric method previously described (Liu *et al*., 2002; Zhishen *et al*., 1999). The results were expressed as mg CE/100 g FW. The total phenolic content was determined using a modified Folin-Ciocalteau colorimetric method, with results expressed as mg GAE/100 g FW (Singleton *et al*., 1999; Liu *et al*., 2002). Antioxidant properties were determined by the ABTS radical scavenging assay, as previously described (Re *et al*., 1999). Results were expressed as Trolox equivalent antioxidant capacity (mM Trolox).

Extraction and HPLC-DAD analysis

Quantification of individual phenolic compounds was performed using reversed phase HPLC analysis. Samples were analyzed using a HPLC Agilent-1200 series (Agilent Technologies, Santa Clara, CA, USA) with UV-Vis DAD detector for multi wavelength detection. After injecting 5 μL of sample, the separation was performed in an Agilent-Eclipse XDB C-18 column (4.6 × 150 mm), which was tempered at 25ºC. Two solvents were used for the gradient elution: A (H_2O + 2% formic acid) and B (80% acetonitrile + 2% formic acid + H_2O). The elution program used was as follows: from 0 to 10 min 0% B, from 10 to 28 min gradually increased 0-25% B, from 28 to 30 min 25% B, from 30 to 35 min gradually increased 25-50% B, from 35 to 40 min gradually increased 50-80% B, and finally for the last 5 min gradually decreased 80-0% B. Phenolic compounds in the samples were identified by comparing their retention times and spectra with retention times and spectra of standards for each component. Quantitative data were calculated from the calibration curves. Content of phenolic compound were expressed as mg/mL, and subsequently recalculated as mg/kg fresh weight.

Statistical analysis

Three factorial experimental design using ANOVA and Tukey's multiple comparison tests were used to analyze the data. The viral status of the plant (PPV-free, PPV-D infected, PPV-Rec infected), harvest stages (HS1, HS2, HS3, HS4) and harvest year (2017, 2018) were taken as the factors of variation. Statistical analysis was performed using Statistica 7 (StatSoft, Inc., Tulsa, OK, USA).

Results

PPV strain detection and characterization

In 2017, IC-RT-PCR analysis revealed that 177 trees were infected with PPV. PPV-Rec strain was detected in 160 trees, PPV-D strain in 17 trees, and in 2 trees mixed-infection were confirmed (PPV-D+PPV-Rec). Based on these results, we selected 10 systematically infected trees (5 per each strain) and 5 PPV-free trees for the main part of the study. The selected trees were again tested in 2018 to confirm particular PPV-strain presence/absence. The results confirmed strain-typing results from 2017. Other viruses (PDV, PNRSV, ACLSV, MLRSV and ApMV) were not detected in any of the selected 15 trees.

Chemical properties of plum fruits

Anthocyanins are being developed and constantly accumulated in plums during ripening. During HS1 in both harvest years, no traces of fruit pigments were observed in all tested samples. Plums infected with PPV-Rec strain possessed the highest amount of pigments, followed by healthy fruits and PPV-D infected samples, while significantly higher amount of anthocyanins were accumulated in plum samples harvested in 2018 than in 2017 (Table S2 [suppl]).

Viral status × harvest stage interaction showed high statistical significance on total anthocyanin content ($p < 0.001$; Table 3). Within the same viral status of the plum, amount of anthocyanins increased with on-tree ripening. Within two initial harvest stages (HS1, HS2), influence of the viral status did not play an important role, resulting in the statis-

Table 3. Three factorial ANOVA: effect of viral status, harvest stage, harvest year and their interaction on phenolic profile and antioxidative capacity of plum fruits.

Compound / class of compounds	A (viral status)	B (harvest stage)	C (harvest year)	A × B	A × C	B × C	A × B × C
Total anthocyanin content	***	***	***	***	***	***	***
Total flavonoid content	ns	***	***	*	ns	***	**
Total phenolic content	ns	***	***	**	ns	***	***
Antioxidant capacity (ABTS)	ns	***	ns	ns	ns	**	*
Neochlorogenic acid	ns	***	***	***	***	***	***
Caffeic acid	ns	***	***	**	ns	***	***
Chlorogenic acid	***	***	***	***	***	***	***
p-coumarolyquinic acid	ns	***	***	*	ns	***	ns
Quercetin rutinoside (rutin)	**	***	***	***	***	ns	***
Quercetin galactoside (hyperoside)	**	***	***	***	***	*	***
Quercetin glucoside (isoquercetin)	**	***	ns	**	***	***	*
Cyanidin 3-*O*-galactoside (idaein)	ns	***	***	ns	ns	***	ns
Cyanidin 3-*O*-glucoside (chrysanthemin)	***	***	***	**	***	***	**
Cyanidin-3-*O*-rutinoside (keracyanin)	***	***	***	ns	**	**	ns

ns, *, **, ***: not significant or significant at $p < 0.05$, 0.01, 0.001, respectively.with PPV-Rec strain, respectively.

tically insignificant differences. Viral influence was highly augmented during the last two stages of ripening, especially in HS4. Namely, the highest level of anthocyanins in PPV-Rec samples (43.6 mg/100 g FW) was followed by healthy (33.5 mg/100 g FW) and finally with PPV-D samples (24.9 mg/100 g FW). Interaction of the viral status and harvest year revealed dominant influence of the PPV-Rec strain within the same harvest year on total anthocyanin content. Moreover, no difference was found between negative and PPV-D samples in 2017. Amount of accumulated pigments in plums with identical viral status or within the same harvest stage was significantly increased in 2018 compared to 2017 (Table S2 [suppl]).

Three-factorial analysis of variance of the anthocyanin content according to viral status, harvest stage and year revealed high influence of virus presence in later harvest stages in rainier harvest year (Fig. 1A). Under such circumstances (HS4, 2018), again the most dyed plums were of PPV-Rec strain (61.6 mg/100 g FW), followed by negative sample (48.0 mg/100 g FW) and PPV-D (31.3 mg/100 g FW). In HS3 samples of 2018, PPV-Rec sample had significantly higher amount of pigments compared to PPV-D and negative plums.

Statistical analysis showed a decrease in level of total flavonoids, total polyphenolics and antioxidant capacity during ripening, being the highest in the initial harvest stage (Table S2 [suppl]). Virus presence had no influence on the contents of these traits, while harvest year played an important role in the contents of flavonoids and polyphenolics, being higher in 2017 (Table 3). Antioxidant capacity showed no changes in two harvest years. Statistical interaction viral status × harvest year revealed no statistical differences in all three measured traits. Interaction viral status × harvest stage revealed no influence on antioxidant capacity, slight influence on total flavonoids content ($p < 0.05$) and higher influence on total phenolics ($p < 0.01$) (Table 3). Within the same harvest stage, virus absence or presence of both strains showed no statistically significant difference in content of total flavonoids or phenolics. Plum ripening influences the total flavonoid content (Fig. 1B). The presence of the virus is the most notable in the third and fourth harvest stage of 2017, since sudden drop was noticed compared to HS1 and HS2 of the same harvest year. No differences in 18HS1 was detected between sample with different viral status (Table S2 [suppl]).

Maturity stage affects the total phenolic content in the plum fruits. Fig. 1C shows different behaviors of plum fruits during 2017 and 2018. Negative sample of HS1 in 2017 showed significantly higher amount of phenolics (574.5 mg/100 g FW), compared to PPV-D infected sample (427.6 mg/100 g FW) and PPV-Rec infected sample (334.9 mg/100 g FW) within the same harvest stage and year. Consequently, the same order is noticed in antioxidant capacity within the same group (HS1, 2017), giving values of 1.51, 1.11 and 1.12 mM Trolox for negative, PPD-D and PPV-Rec samples, respectively. Significant

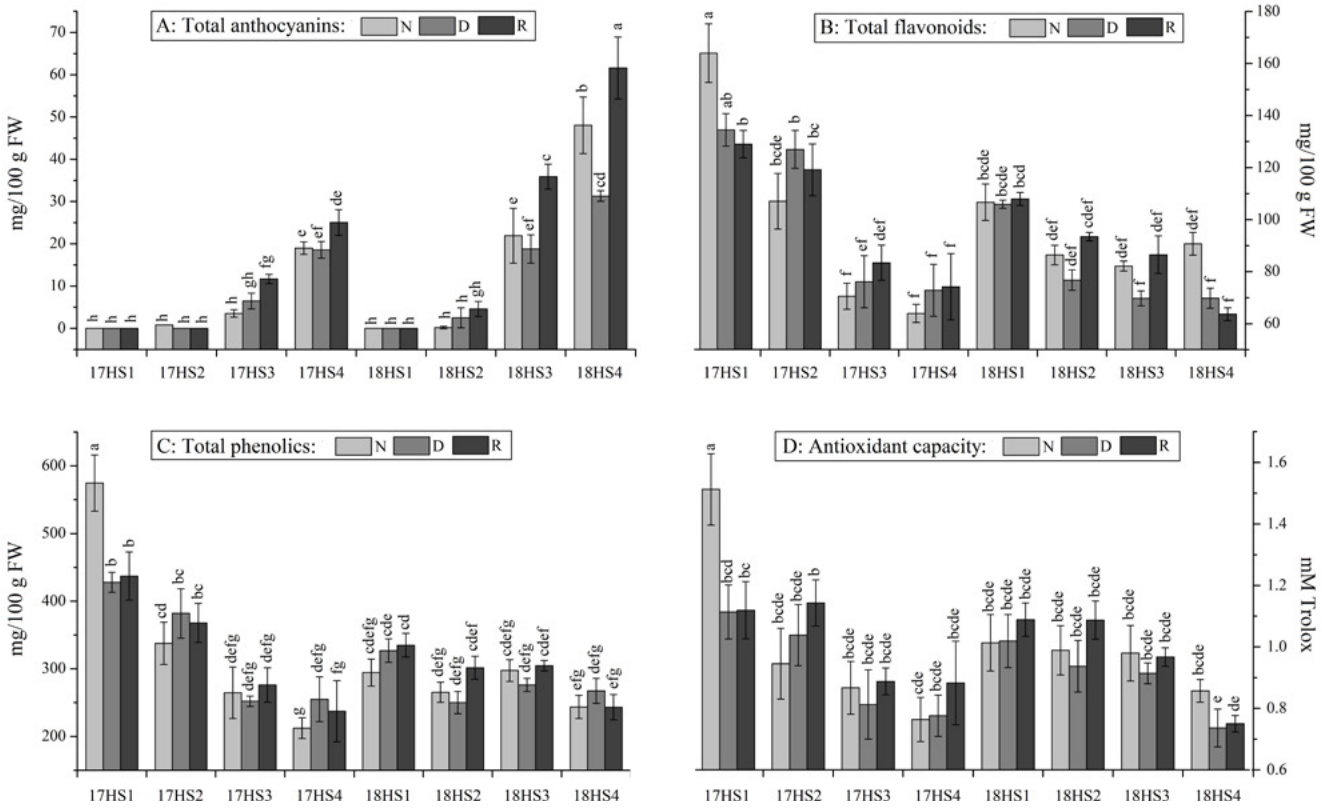

Figure 1. Changes of total anthocyanin content (A), total flavonoid content (B), total phenolic content (C), and antioxidant capacity (D) during ripening in PPV-free and PPV-infected 'Čačanska Lepotica' plums over two successive harvest years. N, D, R stand for negative sample (virus-free), sample infected with PPV-D strain and sample infected with PPV-Rec strain, respectively. Values with different letters within all harvest stages and both harvest years denote statistically significant differences (Tukey's test, $p < 0.05$). Error bars represent standard deviation. ns, *, **, ***: not significant or significant at $p < 0.05$, 0.01, 0.001, respectively.

drop in polyphenolic content was detected in 17HS3 and 17HS4 compared to first two harvest stages, while among them no statistically significant differences was found.

In 2017 total phenolic content decreased during ripening within certain experimental group. For instance, total phenolic content in virus-free samples decreased from 574.5 (17HS1-N) to 337.6 (17HS2-N), then 264.8 (17HS3-N) and 212.3 mg/100 g FW (17HS4-N). It is difficult to withdraw any general conclusion regarding the total phenolic content in 2018. Namely the results presented in Fig. 1C showed that level of polyphenolics did not change during the entire 2018, regardless the viral status and harvest stage of the plum samples.

Changes in antioxidant capacity during 2017 were very similar to those for polyphenolic content (Fig. 1D). Plotting total phenolic content *vs* antioxidant capacity for all plum samples of 2017 harvest year, coefficient of determination, R^2, of 0.924 was calculated. This result suggests strong correlation and emphasizes the main contribution of polyphenolic molecules on antioxidant capacity. Given the unbalanced weather conditions in 2018, especially in the summer period, high correlation between antioxidant capacity and polyphenolic content could not be achieved ($R^2 = 0.514$).

Using HPLC technique, hydroxycinnamic acids, flavonols and anthocyanins were identified and quantified in edible part of 'Čačanska Lepotica' plum fruits, during ripening period of 2017 and 2018, in virus-free samples and samples with PPV infection of both D and Rec strains, and the results are presented in Table 4. Hydroxycinnamic acids were the most abundant class of phenolics and four such acids were identified in 'Čačanska Lepotica' plum samples: neochlorogenic acid, caffeic acid, chlorogenic acid and *p*-coumaroylquinic acid.

Contents of neochlorogenic acid, caffeic acid and *p*-coumaroylquinic was not influenced by the viral status of the plant (Table S2 [suppl]). Virus presence resulted in an increase in chlorogenic acid content in infected samples (2.34 and 2.17 mg/kg FW in PPV-D and PPV-Rec samples, respectively), while the negative samples contained the lowest amount (1.90 mg/kg FW). On-tree ripening resulted in a lowered amount of all four hydroxycinnamic acids, while all samples contained higher amount of these acids in 2017 compared to 2018. Viral status × harvest stage interaction showed high statistical significance on neochlorogenic acid content and chlorogenic acid content ($p < 0.001$), moderate statistical significance on caffeic acid content ($p < 0.01$)

Table 4. Content of selected phenolics in PPV-free and PPV-infected 'Čačanska Lepotica' plums over two successive harvest years, in mg/kg FW.

Compound	Strain	Harvest year	HS1	HS2	HS3	HS4
Neochlorogenic acid	Neg	2017	28.9 ± 0.2 a	21.8 ± 1.6 cd	15.0 ± 1.3 ghij	15.6 ± 0.8 ghij
	PPV-D		26.9 ± 0.5 a	25.9 ± 0.4 ab	19.3 ± 1.0 de	15.5 ± 0.6 ghij
	PPV-Rec		29.0 ± 0.4 a	23.5 ± 0.2 bc	19.1 ± 0.5 def	15.3 ± 0.8 ghij
	Neg	2018	16.3 ± 1.7 efgh	15.8 ± 0.1 fghi	11.5 ± 1.8 k	15.3 ± 2.4 ghij
	PPV-D		16.0 ± 1.9 efghi	13.4 ± 0.8 hijk	12.7 ± 1.0 ijk	12.4 ± 0.3 jk
	PPV-Rec		18.3 ± 1.3 efg	14.0 ± 0.6 hijk	12.3 ± 0.8 jk	11.6 ± 1.2 k
Caffeic acid	Neg	2017	0.55 ± 0.08 a	0.34 ± 0.02 def	0.23 ± 0.01 ghi	0.23 ± 0.01 ghi
	PPV-D		0.44 ± 0.01 bc	0.40 ± 0.02 cd	0.29 ± 0.03 efgh	0.24 ± 0.01 ghi
	PPV-Rec		0.48 ± 0.01 ab	0.35 ± 0.03 de	0.29 ± 0.01 efg	0.22 ± 0.01 ghi
	Neg	2018	0.21 ± 0.01 ghi	0.21 ± 0.01 ghi	0.15 ± 0.01 i	0.21 ± 0.04 ghi
	PPV-D		0.23 ± 0.01 ghi	0.21 ± 0.03 ghi	0.16 ± 0.01 i	0.22 ± 0.05 ghi
	PPV-Rec		0.21 ± 0.01 hi	0.26 ± 0.05 fgh	0.16 ± 0.01 i	0.17 ± 0.03 i
Chlorogenic acid	Neg	2017	4.7 ± 0.1 a	3.2 ± 0.3 cd	2.1 ± 0.2 efg	1.7 ± 0.1 fgh
	PPV-D		4.3 ± 0.4 ab	4.6 ± 0.1 ab	3.3 ± 0.2 cd	2.9 ± 0.2 de
	PPV-Rec		4.3 ± 0.4 ab	3.9 ± 0.1 bc	2.6 ± 0.3 de	2.2 ± 0.1 ef
	Neg	2018	1.3 ± 0.2 ghi	1.1 ± 0.2 hij	0.9 ± 0.2hijk	0.1 ± 0.0 k
	PPV-D		1.3 ± 0.3 ghi	1.2 ± 0.2 hij	0.7 ± 0.1ijk	0.4 ± 0.1 jk
	PPV-Rec		1.1 ± 0.2 hij	1.3 ± 0.1 ghi	0.9 ± 0.1hijk	0.1 ± 0.0 hij
p-coumarolyquinic acid	Neg	2017	0.28 ± 0.07	0.18 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
	PPV-D		0.24 ± 0.01	0.20 ± 0.01	0.15 ± 0.01	0.12 ± 0.01
	PPV-Rec		0.24 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
	Neg	2018	0.11 ± 0.03	0.11 ± 0.01	0.07 ± 0.01	0.08 ± 0.00
	PPV-D		0.10 ± 0.02	0.008 ± 0.01	0.08 ± 0.00	0.08 ± 0.01
	PPV-Rec		0.08 ± 0.01	0.008 ± 0.00	0.07 ± 0.05	0.09 ± 0.00
Quercetin rutinoside (rutin)	Neg	2017	0.18 ± 0.06 b	0.11 ± 0.03 bcde	0.11 ± 0.02 cde	0.11 ± 0.05 bcde
	PPV-D		0.13 ± 0.01 bcde	0.27 ± 0.05 a	0.14 ± 0.04 bcd	0.12 ± 0.01 bcde
	PPV-Rec		0.16 ± 0.01 bc	0.10 ± 0.01 bcde	0.08 ± 0.03 de	0.10 ± 0.02 cde
	Neg	2018	0.13 ± 0.01 bcde	0.12 ± 0.03 bcde	0.09 ± 0.01 cde	0.12 ± 0.02 bcde
	PPV-D		0.11 ± 0.01 bcde	0.11 ± 0.01 bcde	0.08 ± 0.01 de	0.10 ± 0.02 cde
	PPV-Rec		0.11 ± 0.02 bcde	0.13 ± 0.02 bcde	0.12 ± 0.02 bcde	0.06 ± 0.00 e
Quercetin galactoside (hyperoside)	Neg	2017	0.20 ± 0.05 ab	0.12 ± 0.02 cd	0.10 ± 0.03 d	0.14 ± 0.01 bcd
	PPV-D		0.17 ± 0.03 bcd	0.28 ± 0.06 a	0.17 ± 0.05 bcd	0.14 ± 0.01 bcd
	PPV-Rec		0.18 ± 0.02 bc	0.13 ± 0.02 bcd	0.12 ± 0.02 cd	0.12 ± 0.02 cd
	Neg	2018	0.14 ± 0.01 bcd	0.12 ± 0.01 bcd	0.13 ± 0.01 bcd	0.17 ± 0.01 bcd
	PPV-D		0.12 ± 0.04 cd	0.12 ± 0.01 cd	0.11 ± 0.02 cd	0.11 ± 0.01 cd
	PPV-Rec		0.13 ± 0.01 bcd	0.15 ± 0.01 bcd	0.10 ± 0.07 cd	0.10 ± 0.01 cd
Quercetin glucoside (isoquercetin)	Neg	2017	0.067 ± 0.014 ab	0.030 ± 0.009 cde	0.027 ± 0.005 de	0.039 ± 0.006 bcde
	PPV-D		0.045 ± 0.015 bcde	0.066 ± 0.019 ab	0.052 ± 0.010 abcde	0.046 ± 0.010 bcde
	PPV-Rec		0.056 ± 0.013 abcd	0.038 ± 0.002 bcde	0.036 ± 0.004 bcde	0.042 ± 0.002 bcde
	Neg	2018	0.028 ± 0.004 cde	0.026 ± 0.010 de	0.043 ± 0.011 bcde	0.059 ± 0.003 abc
	PPV-D		0.024 ± 0.002 e	0.031 ± 0.003 cde	0.040 ± 0.015 bcde	0.065 ± 0.004 ab
	PPV-Rec		0.026 ± 0.002 de	0.047 ± 0.006 bcde	0.067 ± 0.004 ab	0.081 ± 0.025 a
Cyanidin 3-*O*-galactoside (idaein)	Neg	2017	n.d.	0.03 ± 0.00	0.21 ± 0.09	0.47 ± 0.11
	PPV-D		n.d.	0.10 ± 0.01	0.29 ± 0.07	0.51 ± 0.14
	PPV-Rec		n.d.	0.02 ± 0.02	0.34 ± 0.04	0.58 ± 0.14
	Neg	2018	0.04 ± 0.03	0.49 ± 0.03	1.15 ± 0.03	1.44 ± 0.11
	PPV-D		0.19 ± 0.02	0.33 ± 0.06	0.75 ± 0.02	1.26 ± 0.20
	PPV-Rec		0.29 ± 0.02	0.60 ± 0.09	1.60 ± 0.1	1.64 ± 0.10

Table 4 (cont.). Content of selected phenolics in PPV-free and PPV-infected 'Čačanska Lepotica' plums over two successive harvest years, in mg/kg FW.

Compound	Strain	Harvest year	HS1	HS2	HS3	HS4
Cyanidin 3-*O*-glucoside (chrysanthemin)	Neg	2017	n.d. h	0.08 ± 0.03 gh	0.54 ± 0.13 efgh	1.28 ± 0.16 cd
	PPV-D		n.d. h	0.24 ± 0.00 fgh	0.74 ± 0.01 def	1.24 ± 0.27 cd
	PPV-Rec		n.d. h	0.09 ± 0.02 gh	0.69 ± 0.06 efg	1.27 ± 0.26 cd
	Neg	2018	0.12 ± 0.02 gh	0.49 ± 0.08 fgh	1.55 ± 0.22 bc	1.94 ± 0.14 ab
	PPV-D		0.52 ± 0.04 efgh	0.59 ± 0.09 efg	1.08 ± 0.19 cde	1.25 ± 0.34 cd
	PPV-Rec		0.61 ± 0.06 efg	0.80 ± 0.10 def	2.00 ± 0.21 ab	2.18 ± 0.33 a
Cyanidin-3-*O*-rutinoside (keracyanin)	Neg	2017	n.d.	0.03 ± 0.00	0.14 ± 0.04	0.38 ± 0.05
	PPV-D		n.d.	0.05 ± 0.01	0.17 ± 0.04	0.40 ± 0.03
	PPV-Rec		n.d.	0.03 ± 0.01	0.15 ± 0.02	0.43 ± 0.09
	Neg	2018	0.02 ± 0.00	0.06 ± 0.02	0.23 ± 0.02	0.54 ± 0.15
	PPV-D		0.10 ± 0.04	0.12 ± 0.01	0.31 ± 0.06	0.54 ± 0.06
	PPV-Rec		0.14 ± 0.01	0.11 ± 0.01	0.41 ± 0.01	0.65 ± 0.08

Values with different letters within all harvest stages, both harvest years and compound denote statistically significant differences (Tukey's test, $p < 0.05$). ns, *, **, ***: not significant or significant at $p < 0.05$, 0.01, 0.001, respectively. n.d. – not detected.

and low statistical significance on p-coumarolyquinic acid content ($p < 0.05$) (Table 3). Within the same viral status of the plum, amount of all four hydroxycinnamic acids decreased during on-tree ripening, while within the same harvest stage, viral status did not play an important role. Interaction viral status × harvest year revealed high influence on neochlorogenic acid and chlorogenic acid contents, with a decrease within the same viral status. Statistical interaction harvest stage × harvest year showed a decrease within the same harvest year, and within the same harvest stage. Three-factorial analysis of variance of the hydroxycinnamic acids contents according to viral status, harvest stage and year revealed higher accumulation of neochlorogenic acid and caffeic acid in the first three harvest stages of 2017, compared to the corresponding harvest stages of 2018 (Table 4). No statistically significant differences in content of neochlorogenic acid between 17HS4 and 18HS4 was found, as well as in content of caffeic acid between these two harvest stages. In all corresponding harvest stages between 2017 and 2018 (17HS1-18HS1; 17HS2-18HS2; 17HS3-18HS3; 17HS4-18HS4), higher amount of chlorogenic acid was found in plums of 2017. It seemed that rainier weather conditions resulted in a decrease of individual hydroxycinnamic acids.

Among flavonols, quercetin rutinoside (rutin), quercetin galactoside (hyperoside) and quercetin glucoside (isoquercetin) were identified. Plant's viral status showed certain influence on individual flavonoids contents ($p < 0.01$; Table 3). The lowest amount of rutin and hyperoside in PPV-Rec infected samples was observed. The amount of rutin and hyperoside was decreased during ripening, while the amount of isoquercetin was increased. While harvest year had no influence on the amount of isoquercetin in plum samples, the lower amount of rutin and hyperoside was detected in the samples harvested in 2018 (Table S2 [suppl]). Viral status × harvest stage interaction showed high statistical significance on rutin and hyperoside contents ($p < 0.001$) and moderate statistical significance on isoquercetin content ($p < 0.01$), while viral status × harvest year interaction gave high statistical significance on all three flavonoids ($p < 0.001$) (Table 3). Results of such two-components interaction gave no possibility to draw any clear conclusion (Table S2 [suppl]).

Not so high variations in the content of rutin was detected among all tested plum samples (Table 4). Highest content of rutin was found in 17HS2-D (0.27 mg/kg FW), while the lowest in 18HS4-R (0.06 mg/kg FW). Similar behavior was noticed in the content of hyperoside. It was difficult to draw any conclusion regarding the content of isoquercetin in plums, without any clear trend. Nevertheless, it seemed that harvest year did not play an important role in individual flavonoids contents.

Given the color of plum fruits, expected identification of certain anthocyanins was confirmed: cyanidin 3-*O*-galactoside (idaein), cyanidin 3-*O*-glucoside (chrysanthemin) and cyanidin 3-*O*-rutinoside (keracyanin). Viral status showed no influence on idaein content in plums. The lowest amount of chrysanthemin was detected in negative plum samples, while chrysanthemin was the most abundant in PPV-Rec samples (Table S2 [suppl]). Both harvest stage and harvest year influenced the individual anthocyanins contents. As expected, on-tree ripening resulted in an increase in all three detected anthocyanins, while higher amount of these pigments were noticed in 2018. Viral status × harvest stage interaction showed moderate statistical significance on chrysanthemin content ($p < 0.01$) and no statistical significance on idaein and keracyanin content (Table 3). Within the same viral status, amount of chrysanthemin increased during on-tree ripening. Interaction viral status × harvest year revealed an increase in contents of chrysanthemin and keracyanin within the same viral status during ripening.

The anthocyanins were not detected in 17HS1, while they appeared in the early ripening stage of 2018. As expected, the formation of fruit's pigments was progressed during ripening, reaching the maximum in the last harvest stage in both tested years. The amount of individual anthocyanins was higher in 2018 compared to 2017 in all harvest stages. It is obvious that virus presence had no influence on accumulation of individual anthocyanins, except on chrysanthemin in last two harvest stages of 2018, where PPV-Rec samples were the richer with chrysanthemin (2.00 and 2.18 mg/kg FW for 18HS3 and 18HS4, respectively), compared to PPV-D (1.08 and 1.25 mg/kg FW) and negative sample (1.55 and 1.94 mg/kg FW) (Table 4).

Given the ultimate harvest stage, as the most utilizable in commercial purposes, effect of the PPV infection during 2017 was only noticed in chlorogenic acid content, with PPV-D sample possessing higher amount of this acid (2.9 mg/kg FW), compared to negative (1.7 mg/kg FW) and PPV-Rec infected sample (2.2 mg/kg FW). The amount of remaining nine identified compounds was not altered by the presence of both PPV strains in 2017. Content of neochlorogenic acid and chrysanthemin in the last harvest stage of 2018 was altered by PPV infection. Negative plum sample possessed the highest amount of neochlorogenic acid in 18HS4 (15.3 mg/kg FW), while chrysanthemin was the most abundant in PPV-Rec sample of 18HS4 (2.18 mg/kg FW).

Discussion

Leaf symptoms and premature fruit drops

In this work, ripening of PPV-infected and uninfected 'Čačanska Lepotica' plum trees was followed during two successive years. Leaves on the infected trees expressed typical Sharka symptoms: chlorotic light green rings, patterns and vein clearing. Fruits on all selected trees were normally developed, without any symptoms that might be ascribed to PPV infection. There were no differences on intensity or type of the leaf symptoms between PPV-Rec and PPV-D infected trees. Also, there were no premature fruit drops in any of the trees selected for the analysis – a phenomenon that was reported in sensitive cultivars 'Požegača' and 'Čačanska Rodna'. Premature fruit drop is particularly evident in very sensitive cultivar 'Požegača' (also known as 'Bistrica', 'Madžarka' or 'Kyustendilska sinja sliva') which reaches up to 100% (Németh, 1986; Jevremović, 2013). According to Milosevic *et al*. (2010), about 50% of 'Čačanska Rodna' fruits drop prematurely. Fruits on infected trees ripen earlier, being not deformed and are fully usable for consumption or processing. On the other hand, premature fruit drop does not occur in PPV-tolerant cultivars. 'Čačanska Lepotica' is a cultivar that express PPV symptoms on leaves, but there are no fruit deformations and premature fruit drop due to the viral infection. Based on these characteristics, results of the studies performed in several countries confirmed PPV tolerance of 'Čačanska Lepotica' (Kegler *et al.,* 1998), but there were no studies on the influence of different PPV strains on chemical composition of its fruits.

Viral infection and chemical composition of plum fruits

Weather conditions of two harvest years, 2017 and 2018, regarding the rainfall during summer months, were extremely different (Table 1). Unordinary rainier summer of 2018 influenced a chemical composition of plum fruits to a certain extent, so it was very difficult to distinguish the influence of the plant's viral status from the influence of the weather condition.

Hydroxycinnamic acids were the most abundant class of phenolics found in plums. Usenik *et al*. (2017) found lower content of hydroxycinnamic acids in PPV-infected plums (undeformed and necrotic tissues) compared to the virus-free samples. The same trend was noticed in long term infected plum trees (at least 5 years) during the entire examined ripening period (0, 9 and 22 days of ripening), and in short term infected trees (at least one year) on the 9th and 22nd day of ripening (Usenik *et al*., 2015).

Our results revealed no influence of PPV infection on all hydroxycinnamic acids (neochlorogenic acid, caffeic acid, chlorogenic acid and *p*-coumarolyquinic acid) in all harvest stages during 2018, except the influence on neochlorogenic acid content in 18HS4 (Table 4). More prominent influence of infection was found in few stages of 2017, with additional accumulation of chlorogenic acid in both infected samples of HS3 and HS4 and neochlorogenic acid in HS3. Usenik *et al*. (2015) explained reduced amount of hydroxycinnamic acids in infected samples by stress-induced alteration of biosynthetic pathway of flavonoids. These authors noticed an increased amount of flavonoids and anthocyanins in infected samples, and thus concluded that additional synthesis of flavonoids, on account of reduced synthesis of hydroxycinnamic acids, occurred in infected plum samples. Such conclusion cannot be drawn based on our results, since only during 17HS2 the highest amount of all detected flavonoids (rutin, hyperoside, isoquercetin) was detected in PPV-D infected sample, while no statistically significant differences were found in other harvest stages regarding all detected flavonoids and all detected anthocyanins (idaein, chrysanthemin, keracyanin) in 2017. The later harvest year brought even more interesting results, with additional accumulation of only chrysanthemin in PPV-Rec infected plums during HS3 and HS4, compared to PPV-D and negative sample. Viral status had no influence on contents of other individual compounds in 2018 whatsoever (except neochlorogenic acid in HS4, as above-mentioned). Furthermore, some authors claimed that increased amount of hydroxycinnamic acids in infect-

ed sample might be a sign of plant's response to pathogens (Mikulic Petkovsek *et al*., 2013). It is also known that stress conditions favor the flavonoids and anthocyanins formation in plants (Chalker-Scott, 1999; Usenik *et al*., 2015). Since infection had no influence on concentration of all individual phenolic compounds in HS4 during both harvest years (except chlorogenic acid in 17HS4, and neochlorogenic acid and chrysanthemin in 18HS4), we concluded that 'Čačanska Lepotica' is highly tolerant to PPV. In 2017, plum trees were exposed to virus infection as biotic stress, while in 2018 biotic stress was accompanied by the extreme rainfall as abiotic stress.

Four hydroxycinnamic acids were identified in 'Čačanska Lepotica' plum samples. Neochlorogenic acid ranged from 11.5 to 29.0 mg/kg FW, which is lower compared to the content detected by Usenik (2021) in 'Čačanska Lepotica' cultivar, grown in an organic (302.8 mg/kg FW) and integrated production system (178.5 mg/kg FW). Tomić *et al*. (2019) detected 136.9, 16.2 and 71.0 mg/kg FW of neochlorogenic acid, chlorogenic acid and p-coumarolyquinic acid, respectively, in 'Čačanska Lepotica' plum, which is one order of magnitude higher compared to our results. As for the viral status of the plant, influence of PPV on the content of neochlorogenic acid in 2017 was observed in HS2 and HS3, with increased accumulation in infected plums, which is in contrary with work of Usenik *et al*. (2015), who obtained significantly higher content of neochlorogenic acid in virus-free plum fruits of 'Brkinska češpa' then in PPV-infected samples. On the other hand, the same authors revealed a significant influence of infection on average content of chlorogenic acid, stating that plums with short-term infection and virus-free samples had significantly more chlorogenic acid than plums with long-term infection. Our results showed that, if there was statistically significant difference in chlorogenic acid content (17HS2, 17HS3, 17HS4), negative sample possessed it less than other two samples. According to the results in Table 4, the PPV effect of caffeic acid and *p*-coumarolyquinic acid contents was quite limited.

Anthocyanins are secondary metabolites responsible for fruits color and their accumulation predominantly in skin increases with ripening. These compounds are involved in general plant's antioxidant protection under biotic and abiotic stress situations. Previous studies showed that content of anthocyanins was higher in all infected samples regardless any circumstances (Usenik *et al*., 2015, 2017). In general, virus infection of sensitive cultivars favors an increase in phenolic compounds, particularly for anthocyanins and flavonols (Espinoza *et al*., 2021). Spectrophotometrically determined content of total anthocyanins in 2017 revealed no influence of viral status on total anthocyanins content (Fig. 1A). Regarding 2018, results from Fig. 1A showing higher amount of anthocyanins in PPV-Rec samples in last two ultimate harvest stages. Apparently PPV-Rec infected samples were richer in anthocyanins under heavy rainfall during summer months, but it was not followed by increased amount of flavonoids and polyphenolics, nor lowered content of hydroxycinnamic acids (Figs. 1A, 1B, 1C).

In our samples, three anthocyanins were detected: idaein, chrysanthemin and keracyanin. The most abundant anthocyanins in European plum (*Prunus domestica*) are chrysanthemin and keracyanin, while idaein is mostly present in Japanese plum (*Prunus salicina*) (Jang *et al*., 2018; Tomić *et al*., 2019). No influence of infection on accumulation of these three anthocyanins in 2017, a harvest year with no vast rain stress, gave rise to conclude that 'Čačanska Lepotica' is highly tolerant plum cultivar to PPV. In 2018 PPV-Rec infected plums contained increased amount of chrysanthemin in last two harvest stages.

Total content of flavonoids and phenolics (Figs. 1B and 1C) revealed no influence of virus infection during both 2017 and 2018, except in 17HS1. Since there were no differences among the all samples of 17HS3, 17HS4, 18HS3 and 18HS4, regarding both total flavonoids and total polyphenolics, it seemed that extreme rainfall in 2018 had no influences on the contents of these two traits. For the more valuable conclusion, the effect of the rainfall should be further explored within certain plum groups (virus-free and infected samples), excluding the viral effect on the plums. Since no effect of the virus infection is detected, except in in 17HS1, once again we draw the conclusion of high tolerance of 'Čačanska Lepotica' to plum pox virus. This conclusion is additionally strengthened by the results of Usenik *et al*. (2015, 2017), that detected significantly higher amount of flavonols in infected fruits, particularly in long-term infected plums, as the results of plant's protective mechanism against PPV-infection.

Our results showed three flavonols identified in plum samples: rutin, hyperoside and isoquercetin. Tomić *et al*. (2019) detected rutin in 'Čačanska Lepotica' cultivar (11.0 mg/kg FW), but no traces of hyperoside and isoquercetin were found. On the other hand, all these flavonols were previously detected in plum fruits (Jaiswal *et al*., 2013; Usenik *et al*., 2015). Rutin, hyperoside and isoquercetin varied in healthy plum samples of 'Brkinska češpa' from 5.4 to 16.6 mg/kg FW, 1.7 to 4.2 mg/kg FW and 1.6 to 3.7 mg/kg FW, respectively (Usenik *et al*., 2015), which is higher content of these compounds compared to our results.

Taken into account all these experimental results, it is evident that 'Čačanska Lepotica' can be considered as a highly tolerant plum cultivar to plum pox virus.

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