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Posted Date: 27 December 2023

doi: 10.20944/preprints202312.2039.v1

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Chemical Composition of Healthy and Raspberry Leaf Blotch Emaravirus-Infected Red Raspberry 'Willamette' Fruits

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Abstract: The aim of this study was to determine the changes in chemical composition of the fresh red raspberry 'Willamette' fruits, caused by the presence of raspberry leaf blotch emaravirus (RLBV). In three experimental orchards of 'Willamette' raspberry, fruits were harvested from RLBV–free and RLBV-infected plants in 2019 and 2020. Fruits were collected at appropriate maturity stages and further analyzed on total phenolics, total anthocyanins, and selected individual phenolics. In all three experimental orchards, the phenolic profiles of infected and uninfected fruit samples were considerably different during both studied years. Nonetheless, the intensity of the modifications varied greatly depending on location and harvest year. Statistical analysis revealed that the influence of RLBV infection on the studied features is undeniable, although the influences of weather conditions and soil composition outweigh the influence of RLBV. Taking into consideration all experimental and statistical data, it can be concluded that raspberry 'Willamette' fruits may be RLBV-tolerant, while sensitivity to environmental conditions and soil composition is emphasized.

Keywords: raspberry (*Rubus idaeus* L.); Willamette; RLBV; phenolic profile

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1. Introduction

Red raspberries (*Rubus idaeus* L.) are soft, juicy, aromatic, and extremely perishable fruits that contain numerous secondary metabolites and natural antioxidants with a high free radical scavenging capacity. Raspberries are a rich source of vitamins, minerals, anthocyanins, phenolic acids, and other flavonoids, whose consumption has numerous human health benefits [1–3].

Red raspberry is economically the most important berry fruit in the fruit production of Serbia. According to the Food and Agriculture Organization of the United Nations (FAO), the world's and Serbia's average annual raspberry production in the period 2017 – 2021 is 860.238 t and 117.215 t, respectively [4]. The predominant raspberry cultivar in Serbia is 'Willamette' with a production share of about 90% [5]. 'Willamette' is an American midsummer floricane cultivar with medium-sized fruits. It is well adapted to Serbian agroecological conditions and achieves high yields and top fruit quality. Fruits are particularly suitable for deep freezing, and frozen fruits are one of the leading export commodities of Serbian agriculture.

More than 30 viruses and virus-like agents infect red raspberry and other *Rubus* species [6]. Some viruses cause asymptomatic infection or mild symptoms on leaves. On the contrary, others can induce severe symptoms that can lead to decreased yields and low fruit quality. The most common symptoms found on raspberry leaves in Serbian raspberry orchards are yellow patches and blotches

[7]. Until the discovery of RLBV, these symptoms were described as infestation by the raspberry leaf and bud mite (*Phyllocoptes gracillis* Nalepa) [8]. RLBV is a new negative-strand RNA virus that belongs to the genus *Emaravirus*. Its genome is 17,410 nucleotides (nt) long and consists of eight segmented RNAs [8,9]. The suspected vector of the virus is *P. gracillis* [9–11]. RLBV is widely present in raspberry orchards in Serbia and induces severe symptoms in infected plants [7]. RLBV has been reported on a dozen raspberry cultivars in European countries: Bulgaria, Bosnia and Herzegovina, Finland, Great Britain, Montenegro, Poland, Serbia, Slovakia and Ukraine [11]. The results of the recent study confirmed that RLBV significantly decreases fruit size and weight (up to 27.5%) of 'Willamette' fruits. Also, no significant changes were evidenced in soluble solids content (SSC), titratable acidity of raspberry juice, pH, or total sugar content [12].

The aim of the present study was to assess the chemical composition of RLBV-infected and RLBV-free 'Willamette' fruits.

2. Materials and Methods

2.1. Fruit Sampling

The study was done in three raspberry 'Willamette' orchards in Western Serbia throughout 2019 and 2020: Cerova (43°44.662′ N, 20°6.937′ E, 336 m altitude), Bedina Varoš (43°33.776′N 20° 14.313′E, 686 m), and Devići (43°25.667′N 20° 22.999′E, 942 m). Raspberries in all orchards were trained in the linear system with a planting distance of 2.2 × 0.25 m. Raspberries were trained to wire trellis, which is a common system in practice. In each orchard, 6–8 floricanes per row meter were selected and tied to the wire, forming a trellis. An integrated pest and disease control strategy was used to maintain all orchards. Acaricides were used to control the raspberry leaf and bud mite in accordance with an insect and disease spray schedule.

Twenty floricanes with leaf blotch symptoms and twenty asymptomatic floricanes were randomly selected from each orchard in 2019 and 2020. Each year, 120 canes were selected from three orchards (Table 1).

Table 1. Location and virus presence indication for raspberry 'Willamette' fruits harvested in 2019 and 2020.

Location	Harvest year			
	2019		2020	
	RLBV +	RLBV –	RLBV +	RLBV –
Bedina Varoš	B1+	B1–	B2+	B2–
Devići	D1+	D1–	D2+	D2–
Cerova	C1+	C1–	C2+	C2–

For 2019 and 2020, average monthly and yearly values of air temperature (°C) and precipitation (mm) were collected from automatic weather sensors positioned in close proximity to orchards (Table 2). The data was taken from Serbia's official state hydrometeorological service.

Table 2. Average monthly values of air temperature and precipitation for the investigated period (2019–2020).

Location		Average air temperature (°C)		Average precipitation (mm)	
		2019	2020	2019	2020
April	Bedina Varoš	11.7	10.5	166.8	70.0
	Devići	7.7	6.9	57.9	42.1
	Cerova	11.7	10.1	101.9	22.0
May	Bedina Varoš	13.1	14.1	110.2	197.2
	Devići	9.6	11.3	84.4	67.9
	Cerova	13.2	14.4	176.3	99.0
June	Bedina Varoš	20.4	17.7	328.4	231.0

	Devići	17.2	14.3	126.8	112.9
	Cerova	20.7	18.3	110.5	137.3
July	Bedina Varoš	20.1	19.4	107.2	17.4
	Devići	16.8	16.5	89.1	74.4
	Cerova	20.3	20.0	80.8	84.8
August	Bedina Varoš	21.3	20.6	71.2	46.6
	Devići	17.8	16.9	9.3	265.0
	Cerova	21.2	20.5	68.0	154.4
September	Bedina Varoš	16.5	17.2	2.4	49.6
	Devići	13.2	13.6	54.7	52.8
	Cerova	15.9	16.6	22.6	9.9
October	Bedina Varoš	12.4	11.8	5.4	152.6
	Devići	8.6	8.4	24.8	80.6
	Cerova	11.3	11.1	32.5	64.4

2.2. Agrochemical Characteristics of Soil

Soil samples from three raspberry orchards were adequately taken in order to obtain representative samples and were further analyzed. Soil pH was measured in 1M KCl solution by the standard ISS method (SRPS EN ISO 10390; Institute for Standardization of Serbia). The content of humic substances in soil samples was determined by the standard method ISO 12782–4, while nitrogen was measured by the Kjeldahl method [13]. The determination of the total carbonates, expressed as %CaCO3, was carried out by the titrimetric procedure based on the dissolution of soil carbonates in acid and the subsequent reaction of CO_2 with NaOH [14]. The soil samples were analyzed for accessible phosphorus (expressed as mg P_2O_5/100 g soil) and potassium (expressed as mg K_2O/100 g soil) [15].

2.3. RT-PCR Analysis

All leaf samples (60 symptomatic and 60 asymptomatic) taken from selected canes were tested for the presence of RLBV using reverse-transcription polymerase chain reaction (RTPCR). A modified CTAB procedure was used for total nucleic acid (TNA) extraction, as described by Li et al. [16]. For RLBV detection, two-step RT was done with random hexamer primers [pd(N)6] and Maxima Reverse Transcriptase (Thermo Scientific, USA). PCRs were done with the RLBV-specific primer pair 1287/1095 that amplifies the 567 base-pair (bp) fragment of the nucleocapsid of RNA3 [8]. Amplified products were analyzed by 1.5% agarose gel electrophoresis. All samples were also examined for the presence of the following viruses which infect raspberries in Serbia: raspberry bushy dwarf virus (RBDV), raspberry leaf mottle virus (RLMV), raspberry vein chlorosis virus (RVCV), black raspberry necrosis virus (BRNV), and Rubus yellow net virus (RYNV). The analysis was performed to confirm the infection of symptomatic canes with RLBV and to exclude the possibility of the tested viruses influencing mixed infections.

2.4. Extraction and Determination of Total Anthocyanins and Total Phenolics

Raspberry samples (~ 150 g) were poured into liquid nitrogen and ground in a stainless-steel blender. A powdered sample (10 g) was mixed with 50 mL of 96% ethanol and ultrasonicated. After 30 min of extraction, the mixture was centrifuged two sequential times for 15 min at 3500 rpm, and the supernatant was filtered through a 0.45 mm Minisart filter before analysis. In total, six extractions were performed for every harvest year: three extractions from RLBV infected fruits and three extractions from RLBV-free samples. The obtained extracts were used for the determination of total phenolic content [17] and individual polyphenolic profiles. In order to obtain an extract for anthocyanin content the identical extraction procedure was repeated, but with 50 mL of 96% ethanol/HCl (85:15 v/v) [17)]. All these determinations were performed in triplicate, and results were presented as the mean value of three measurements ± standard deviation.

 doi:10.20944/preprints202312.2039.v1

2.5. HPLC-DAD Analysis

As previously published [18], specific phenolic components were quantified using reversed phase HPLC analysis (Agilent Technologies, Santa Clara, CA, USA). The retention times and spectra of phenolic compounds were compared to those of the standards, and quantification was based on the calibration curves and peak areas. The results were obtained in mg/mL and then reported as mg/100 g or mg/kg of fresh weight.

2.6. 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 (RLBV-infected, RLBV-free), harvest location (Bedina Varoš, Devići, Cerova) and harvest year (2019, 2020) were taken as the factors of variation. Principal component analysis (PCA), based on the content of 11 individual phenolic compounds and 2 groups of compounds, was performed, and PCA was designed. Statistical analyses were performed using Statistica 7 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. RLBV Detection

A total of 120 raspberry leaf samples were tested for RLBV presence by RT-PCR. The analysis confirmed RLBV presence in all samples with leaf blotch symptoms (60 samples, 20 per orchard) and confirmed that RLBV was not detected in all analyzed asymptomatic samples (60 samples, 20 per orchard). All samples were free from other tested viruses (RBDV, RLMV, RVCV, RYNV, and BRNV), excluding their influence on examined traits.

3.2. Chemical Properties of Raspberry Fruits

Using the HPLC technique, 11 compounds were detected in all raspberry samples: 4 phenolic acids (caffeic acid (CA), p-coumaric acid (pCOU), ferulic acid (FA), ellagic acid (EA)), 3 flavonol glycosides (quercetin 3-O-rutinoside (rutin, RUT), quercetin 3-O-glucoside (isoquercetin, ISO-Q), quercetin 3-O-rhamnoside (Q3-RHA)), 2 flavonol aglycones (quercetin (Q), kaempferol (KAE)), and 2 anthocyanins (cyanidin 3-O-glucoside (chrysanthemin, CY3- GLU), cyanidin 3-O-sophoroside (CY3-SOP)) (Table 3). All these compounds were previously detected in raspberry fruits of various cultivars [1,19,20]. Pavlović et al. [21] determined the chemical composition of 'Willamette' raspberry fruits grown at three localities in Serbia and obtained results for caffeic acid (2.45 – 10.37 mg/kg fw) and *p*-coumaric acid (3.26 – 5.79 mg/kg fw) are in great agreement with our results (3.8 – 11.9 and 1.7 – 26.3 mg/kg fw, respectively), while certain discrepancy in the contents of rutin (up to 0.50 mg/kg fw) and kaempferol (up to 0.20 mg/kg fw) was noticed (33.4 – 145.9 mg/kg fw and 3.6 – 18.1 mg/kg fw, respectively). A similar phenolic profile of 'Willamette' raspberries was obtained by other researchers [22,23].

Table 3. Content of individual phenolic compounds, total phenolics (PHENOL) and total anthocyanins (ANTHO) in RLBV-free and RLBV-infected 'Willamette' raspberries over two successive years in three localities.

Compounds/ class of compounds	Bedina Varoš				Devići				Cerova			
	2019		2020		2019		2020		2019		2020	
	RLBV +	RLBV –	RLBV +	RLBV –	RLBV +	RLBV –	RLBV +	RLBV –	RLBV +	RLBV –	RLBV +	RLBV –
CA	9.2 ± 1.6 bc	5.8 ± 1.0 de	3.8 ± 0.3 e	4.6 ± 0.6 e	8.6 ± 0.7 bc	10.2 ± 0.7 ab	5.9 ± 0.7 de	5.2 ± 0.8 e	11.9 ± 0.7 A	11.9 ± 0.2 a	7.7 ± 0.1 cd	7.9 ± 0.3 cd
*p*COU	4.1 ± 1.1 ef	1.7 ± 0.5 f	26.3 ± 1.5 a	7.9 ± 0.2 c	6.4 ± 0.4 cde	6.9 ± 0.3 cd	4.1 ± 0.2 ef	4.3 ± 0.2 def	12.6 ± 1.8 b	8.6 ± 1.5 c	1.8 ± 0.3 f	2.3 ± 0.3 f
FA	10.7 ± 1.6 b	6.4 ± 0.7 cd	8.3 ± 0.9 c	4.9 ± 1.0 d	12.1 ± 0.6 b	11.1 ± 0.8 b	6.0 ± 0.3 d	6.1 ± 0.3 cd	5.6 ± 0.6 d	17.5 ± 0.6 a	5.8 ± 0.8 d	6.6 ± 0.6 cd

 doi:10.20944/preprints202312.2039.v1

EA	194.2 ± 10.0 f	118.6 ± 6.3 h	212.8 ± 8.0 ef	162.3 ± 7.3 g	130.1 ± 4.0 h	106.3 ± 5.9 h	248.3 ± 10.5 d	358.2 ± 8.4 c	222.3 ± 15.5 E	198.3 ± 6.5 ef	502.4 ± 10.9 a	434.0 ± 6.6 b
RUT	145.9 ± 5.6 a	92.9 ± 6.1 c	142.7 ± 6.4 a	33.4 ± 1.9 f	71.4 ± 3.2 d	69.5 ± 3.9 de	54.6 ± 3.8 e	67.7 ± 5.7 de	124.2 ± 7.8 b	91.5 ± 6.3 c	116.5 ± 5.3 b	83.1 ± 5.9 cd
ISO-Q	50.7 ± 3.0 c	35.6 ± 2.5 e	57.5 ± 2.3 b	32.4 ± 1.9 ef	37.1 ± 1.5 de	29.6 ± 1.5 f	54.3 ± 2.6 bc	78.0 ± 2.1 a	36.6 ± 0.9 e	17.1 ± 1.0 g	72.6 ± 2.0 a	42.5 ± 1.7 d
Q3-RHA	63.2 ± 0.9 c	64.9 ± 1.0 c	82.8 ± 2.1 a	79.8 ± 0.8 a	39.7 ± 1.4 e	35.3 ± 2.7 e	22.6 ± 1.3 g	57.2 ± 1.1 d	58.4 ± 1.5 d	70.2 ± 1.6 b	72.2 ± 1.3 b	27.9 ± 1.5 f
Q	6.6 ± 0 .5 c	11.6 ± 1.3 b	46.0 ± 1.7 a	47.3 ± 1.3 a	10.3 ± 0.7 b	5.2 ± 0.5 c	5.5 ± 0.4 c	5.3 ± 0.4 c	6.2 ± 0.4 c	5.8 ± 0.5 c	5.0 ± 0.3 c	5.3 ± 0.2 c
KAE	18.1 ± 1.7	16.0 ± 0.5	12.5 ± 0.6	11.9 ± 1.1	12.6 ± 0.7	12.2 ± 0.6	6.9 ± 0.4	5.9 ± 0.6	10.1 ± 0.8	8.3 ± 0.4	3.6 ± 0.4	3.7 ± 0.2
CY3-GLU	132.8 ± 7.6 a	69.9 ± 6.8 c	15.1 ± 1.7 f	56.5 ± 3.6 d	62.4 ± 4.5 cd	51.4 ± 4.4 d	20.5 ± 1.1 f	22.3 ± 0.8 f	105.8 ± 5.5 b	107.6 ± 3.1 b	35.1 ± 1.3 e	36.3 ± 1.3 e
CY3-SOP	594.4 ± 6.2 a	304.0 ± 6.3 d	95.2 ± 4.4 i	186.1 ± 5.3 f	304.7 ± 6.0 d	238.3 ± 6.9 e	87.3 ± 2.4 i	123.7 ± 4.8 h	503.0 ± 8.6 b	460.8 ± 5.0 c	124.0 ± 4.5 h	167.3 ± 5.4 g
PHENOL	259.8 ± 6.0 e	303.5 ± 0.4 bc	267.6 ± 3.2 de	286.9 ± 7.5 cd	337.9 ± 15.2 a	296.9 ± 5.5 bc	255.1 ± 8.0 e	233.6 ± 3.0 f	315.7 ± 6.9 b	340.9 ± 8.1 a	300.2 ± 1.4 bc	273.8 ± 3.6 de
ANTHO	68.9 ± 15.1 ef	102.6 ± 7.2 b	87.2 ± 3.7 bcde	72.3 ± 1.1 def	103.3 ± 4.5 b	78.8 ± 16.3 cde	65.4 ± 0.6 ef	54.9 ± 0.4 f	98.3 ± 7.1 bc	134.1 ± 6.4 a	94.4 ± 0.7 bcd	73.5 ± 1.2 def

Contents of CA, *p*COU, FA, RUT, Q3-GLU, Q3-RHA, Q and KAE are given in mg (kg fw) $^{-1}$, and contents of CY3-GLU, C3-SOP, PHENOL and ANTHO are given in mg/100 g fw. Different letter in the same raw denote statistically significant difference (Tukey's test, $p < 0.05$) among raspberry fruits of different viral status/locality/year combinations.

3.3. Influence of RLBV-infection, Harvest Year and Locality on Polyphenolic Profile of Raspberry Fruits

Three-factorial ANOVA and Tukey's multiple comparison tests were used to analyze the influence of certain factors on the polyphenolic profile of raspberry fruit samples. Factors of variation (viral status, locality, harvest year) and their interactions are presented in Table 4. It is noteworthy that the influence of harvest year and locality might be actually understood as the influence of weather conditions and soil composition, respectively. Viral infection showed the least effect on the contents of CA, Q3-RHA, Q, PHENOL, and ANTHO (no statistical differences), while such influence was the most dominant on the contents of *p*COU, EA, RUT, ISO-Q, and CY3-SOP ($p < 0.001$). The contents of all detected compounds are highly influenced by harvest year ($p < 0.001$ for all compounds, except *p*COU and Q3-RHA, which are $p < 0.01$) and locality ($p < 0.001$) (Table 4 & Appendix).

Table 4. Three factorial ANOVA: effect of viral status, harvest year, locality and their interaction on phenolics profile of raspberry fruits.

Compounds/ class of compounds	A (viral status)	B (harvest year)	C (locality)	[A×B]	[A×C]	[B×C]	[A×B×C]
CA	ns	***	***	ns	*	ns	***
*p*COU	***	**	***	***	***	***	***
FA	*	***	***	***	***	***	***
EA	***	***	***	***	***	***	***
RUT	***	***	***	***	***	***	***
ISO-Q	***	***	***	*	***	***	***

Q3-RHA	ns	**	***	***	***	***	***
Q	ns	***	***	ns	***	***	***
KAE	**	***	***	ns	ns	ns	ns
CY3-GLU	**	***	***	***	**	***	***
CY3-SOP	***	***	***	***	***	***	***
PHENOL	ns	***	***	***	***	***	***
ANTHO	ns	***	***	***	***	***	***

ns, *, **, ***: not significant or significant at $p < 0.05$, 0.01, 0.001, respectively.

Comparing all infected and all healthy samples, regardless of the locality and harvest year, Figure 1 represents the influence of the viral infection on the polyphenolic profile of raspberry fruit samples. It is obvious that in most of the cases, viral infection prompted either an increase in the content of certain polyphenolics (*p*COU, EA, RUT, ISO-Q, KAE, CY3-GLU, CY3-SOP) or the concentrations remained intact (CA, Q, PHENOL, ANTHO). Only the content of FA decreased after infection. The same behavior was observed when analyzing the dual interaction between *viral status* × *harvest year* (A × B) and *viral status* × *locality* (A × C).

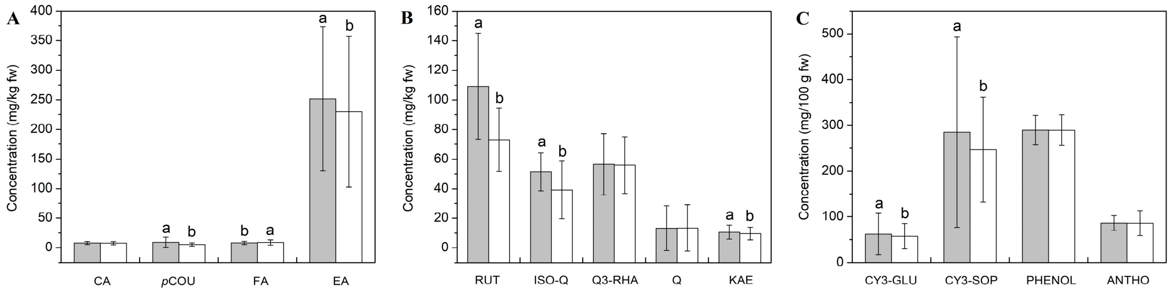

Figure 1. Influence of the RLBV on the phenolic profile: (A) phenolic acids, (B) flavonols, and (C) individual and total anthocyanins and total phenolics. For each compound or class of compounds, different letters indicate significant differences ($p < 0.05$) between RLBV-infected and uninfected fruits. Grey and white rectangles represent RLBV-infected and RLBV-free samples, respectively.

To present it in a more illustrative manner, PCA was applied to determine how infected and healthy raspberry samples of the 'Willamette' in different orchards were grouped based on concentrations of 11 individual compounds and 2 classes of compounds (Figure 2). The first two principal components explain 60.02% (39.03% and 20.99%, respectively) of the total variance. PCA analysis revealed no segregation whatsoever regarding the viral status of the plant. Furthermore, it is evident that infected and uninfected pairs (rounded in Figure 2) stand in close proximity.

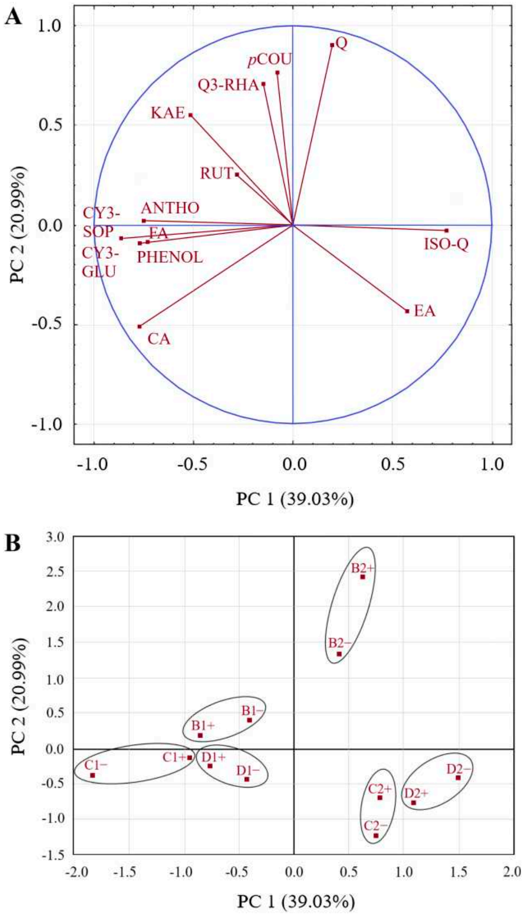

Figure 2. Segregation of 12 raspberry samples according to their polyphenolic profile (11 individual compounds and two groups of compounds) determined by principal component analysis (PCA). (A) Variable loadings; (B) Sample scores. For abbreviations, see section 'Materials and methods'.

The influence of harvest year can be more clearly observed in *viral status* × *harvest year* (A × B) and *harvest year* × *locality* (B × C) interactions. Analyzing the B × C interaction revealed that within the same locality, the content of EA was higher in 2020 compared to 2019, while content of FA was decreased in 2020 (Appendix). Anthocyanins were highly influenced by weather conditions, so the contents of both detected anthocyanins (CY3-GLU and CY3-SOP) were higher in 2019 than in 2020, in all three experimental orchards. For instance, contents of CY3-GLU in raspberry samples harvested in Bedina Varoš, Devići and Cerova were 101.4, 56.9 and 106.7 mg/100 g fw in 2019, and 35.8, 21.4 and 35.7 mg/100 g fw in 2020, respectively. Flavonols did not show any conclusive trend. Interaction A × B showed very similar trend within the same viral status, meaning that weather conditions highly affect the formation of certain polyphenolics. The influence of the weather conditions observed through the harvest years, can be more clearly seen in PCA. All raspberry samples harvested during 2019 are grouped on the negative side of PC1, while 2020-harvested samples formed the other group on the positive side of the same axis (Figure 2). This separation might be attributed to the different weather conditions in 2019 and 2020 in all orchards, which is clearly seen in Table 2. While the average monthly air temperatures were similar in both examined years, the rainfall amounts significantly differed in almost all examined months (April–October). Cheng et al. [24] proved that anthocyanins were more readily accumulated in the skin of grapes (*Vitis vinifera* L.) if the soil contained less water. On the contrary, Li et al. [25] showed that the contents of almost all anthocyanins were increased in the rain-shelter cultivated wine grapes.

The effect of the locality on the polyphenolic profile cannot be clearly seen through dual interactions: *viral status* × *locality* (A × C) and *harvest year* × *locality* (B × C). It turned out that PCA showed good separation among raspberry samples (Figure 2). Namely, the different behavior of

 doi:10.20944/preprints202312.2039.v1

individual polyphenolics in Bedina Varoš locality compared to Devići and Cerova orchards can be clearly observed by PCA. Raspberry samples harvested in Bedina Varoš locality formed the first group, situated on the positive side of PC2. A second group, situated on the negative side of PC2, was formed by the fruit samples harvested in the remaining two orchards, Devići and Cerova. Such a trend can be attributed to the different soil compositions of three orchards (Table 5). Namely, it is clear that contents of phosphorus and potassium in the soil of Bedina Varoš orchard are significantly higher compared to Devići and Cerova orchards, which certainly influence the polyphenolic profile of grown fruits [26]. The content of phosphorus is 27.7-fold and 5.6-fold higher in Bedina Varoš orchard than in Devići and Cerova orchards, respectively. Furthermore, the weather conditions in 2019 and 2020 in Bedina Varoš were quite different compared to the remaining two orchards, especially regarding the rainfall amounts (Table 2). The polyphenolic profile of raspberry fruits appears to be more influenced by weather and soil characteristics than by RLBV infection. Given the wide range in soil composition, particularly in terms of phosphorus and potassium content, and rainfall amounts (for example, the amount of rainfall in the Devići locality in August 2020 was 5.7 times and 1.7 times higher than that in Bedina Varoš and Cerova, respectively), it is not surprising. Delgado et al. [27] proved that anthocyanin content in 'Tempranillo' grapes at veraison increased in proportion to the potassium dose applied in fertilization. Also, increased accumulation of polyphenols in grape fruits with no nitrogen fertilization was observed, while this trend diminished as the potassium fertilization dose was increased. In contrary, purple-blue potatoes showed the highest anthocyanins content with the highest nitrogen fertilization and a lack of phosphorus and potassium fertilization [28]. It seems that every fruit and vegetables species and cultivar require certain level and ratio of nitrogen, phosphorus, and potassium contents in the soil to maximize polyphenolic content.

Table 5. Chemical composition of soil samples taken from the raspberry orchards.

Locality of orchard	pH (in 1M KCl)	$CaCO_3$ (%)	Humic substances (%)	Total nitrogen (%)	Accessible phosphorus (mg P_2O_5/100 g)	Accessible potassium (mg K_2O/100 g)
Bedina Varoš	4.08	0.5	6.04	0.30	40.45	61.4
Devići	4.15	0.5	6.07	0.30	1.46	26.2
Cerova	5.02	0.0	3.33	0.17	7.28	16.3

Polyphenolic compounds belong to the group of secondary metabolites, responsible for defense mechanisms in plants, primarily towards the microbiological infections, including viral infections. The contents of these compounds in fruits depends on various factors, such as fruit species, variety, maturity stage, soil substrate, plant health status, agricultural practices, etc. [29–31]. In this research, the influence of the cultivar was excluded since only 'Willamette' was utilized for analyses. On the other hand, three influences were included: viral status, harvest year (weather conditions), and locality (soil composition). It is very difficult to isolate only the effect of infection on the content of polyphenolics and to draw any clear conclusion or trend regarding the influence of the virus presence. Statistical analyses showed an evident influence of locality and harvest year since PCA clearly differentiated all samples harvested in the first harvest year from the samples harvested the following year, as well as all samples harvested in Bedina Varoš locality from all samples harvested in the remaining two orchards (Figure 2).

It is well known that stress conditions, such as the presence of pathogens, might cause alteration in the contents of flavonoids, anthocyanins, and hydroxycinnamic acids, as a sign of the plant's response to infection [32–34]. Such influence is most obvious in the Bedina Varoš locality, where all four hydroxycinnamic acids, as well as RUT and Q3-GLU, were additionally deposited in fruits as a consequence of infection during both harvest years (Table 3). Some authors claimed that an increased amount of hydroxycinnamic acids is a sign of a plant's response to pathogens [31]. On the other hand, Usenik et al. [32] found a lower content of hydroxycinnamic acids in PPV-infected plums compared to the healthy samples. Such an effect was explained by stress-induced alteration of the biosynthetic pathway of flavonoids, and thus an increased amount of flavonoids was synthesized on account of reduced synthesis of hydroxycinnamic acids in infected plum samples. In our group's research on PPV influence on phenolic compounds, it was noticed that none of the two suggested options occurred, giving rise to the conclusion that the examined plum cultivar could be sorted as highly tolerant towards the virus [18]. In this research, it cannot be concluded that cv 'Willamette' is tolerant towards RLBV, but it is also unclear the effect that such a virus caused. The effect of RLBV was indisputable, but this viral effect on polyphenolic compounds cannot be isolated from other effects in our designed experimental setup. Jevremović et al. [12] concluded that locality played an important role in raspberry polyphenolic profiles when infected by RLBV.

Viral infection had no impact on CA, Q3-RHA, Q, PHENOL and ANTHO (non-significant), then a medium impact on FA ($p < 0.05$), KAE, and CY3-GLU ($p < 0.01$), while a high influence on *p*COU, EA, ISO-Q, and CY3-SOP was detected ($p < 0.001$) (Table 4, Figure 1). Taking this into consideration, it could be concluded that virus presence definitely showed a certain influence on polyphenolic compounds. Nevertheless, such influence was certainly limited and prevailed due to the effects of locality and weather conditions. Since these two parameters played an important role, the different behavior of some phenolic compounds in two successive harvest years might be explained by various weather conditions (for instance, the content of CY3-SOP in raspberry samples harvested in 2019 in Bedina Varoš increased due to the infection from 304.0 to 594.4 mg/100 g fw, while in 2020 it decreased from 186.1 to 95.2 mg/100 g fw. On the other hand, since contents of some compounds showed different trends in various orchards during the same harvest year, such behavior could be due to the different localities and also different weather conditions (for instance, content of CY3-GLU in raspberry samples harvested in Bedina Varoš in 2019 increased as a result of infection from 69.9 to 132.8 mg/100 g fw, while during the same harvest year content of this anthocyanin remained intact after infection in the other two localities). Apparently, the influence of viral status was overpowered by the influence of weather and locality on the phenolic profile of raspberry 'Willamette'. On the other hand, Jevremović et al. [12] concluded that RLBV significantly decreased the dimensions and weight of infected raspberry 'Willamette' fruits in the same three orchard's localities during 2019. The decreases in fruit length, width, and height were 4.01 – 9.8%, 4.06 – 9.47% and 5.88 – 14.9%, respectively, while the decreases in fruit weight due to RLBV infection ranged from 9.15 – 27.49%. Regardless of the orchard locality, RLBV presence, without exception, causes a decrease in the fruit's dimensions and weight. It is clear that RLBV infection overpowers the effects of environmental conditions on 'Willamette' productivity. As for the polyphenolic profile, viral infection was suppressed by environmental conditions, which is clearly demonstrated by PCA. This information could be of great interest to 'Willamette' producers.

4. Conclusions

Based on the findings, it can be concluded that 'Willamette' raspberry fruits cannot be indisputably considered tolerant to RLBV. The influence of RLBV infection is quite minor compared to the influence of soil composition and weather conditions on the chemical composition of fruits. Since weather conditions are annually altered and unpredictable, such influence will certainly prevail on the RLBV effect on raspberry fruits. Soil composition, especially the content of nitrogen, phosphorus, and potassium decreases over the years of fruit growth and can be radically changed by fertilization. These two influences will always overpower the RLBV infection effect on the chemical composition of 'Willamette' fruits and cover it, as clearly shown by PCA. Such information is of great importance to the raspberry producers. As already suggested, growing raspberries in a protected environment could eliminate soil and weather influences and isolate the RLBV infection's influence on the chemical composition of fruits.

Supplementary Materials: Table S1: Influence of the main variables and their interaction on polyphenolic profile of raspberry fruits.

Author Contributions: Conceptualization, N.M. and D.J.; methodology, N.M. and D.J.; software, M.M., B.P. and M.P.; validation, M.M., B.P., B.V. and V.K.; formal analysis, M.P., B.V. and V.K.; data curation, M.M.; writing—

original draft preparation, N.M. and D.J.; writing—review and editing, B.P., M.P. and D.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, grant number 451-0347/2023-01/200088, 451-03-47/2023-01/200124 and 451-03-47/2023-01/200215.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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