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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 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 in terms of total phenolics, total anthocyanins, and selected individual phenolics. In all three experimental orchards, the phenolic profiles of the infected and uninfected fruit samples were considerably different during both studied years. Nonetheless, the intensity of the modifications varied greatly depending on the location and harvest year. Statistical analysis revealed that the influence of RLBV infection on the studied features was undeniable, although the influences of weather conditions and soil composition outweighed the influence of RLBV. Taking into consideration all the experimental and statistical data, it can be concluded that RLBV had an impact on the phenolic profile of raspberry 'Willamette' fruits, 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, the consumption of which 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 was 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. The 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 the fruit size and weight (up to 27.5%) of 'Willamette' fruits. Also, no significant changes were evidenced in terms of the soluble solids content (SSC), titratable acidity of raspberry juice, pH, or total sugar content [12]. As for the polyphenolic profile of raspberry fruits, it is well known that such a profile is highly influenced by various factors, i.e., cultivar specificities, maturity stage, agro-technical treatments, the geographical locality of the orchard, and storage treatment. Numerous studies regarding the influence of these parameters on the polyphenolic content of fresh raspberry fruits have been published [13–16]. Nevertheless, there is only one study dealing with the influence of virus presence in the plant on the chemical composition of raspberry fruit. Malowicki et al. [17] investigated the influence of raspberry bushy dwarf virus (RBDV) on the chemical composition in RBDV-resistant transgenic (RBDV-free) and wild-type 'Meeker' plants (RBDV-infected). The authors confirmed that there were no differences in the concentrations of 30 volatile compounds examined between RBDV-free and RBDV-infected plants. On the other hand, the RLBV influence on the polyphenolic profile of raspberry fruits has not been evaluated up to date. Therefore, 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

This study was conducted 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 a 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 raspberry leaf and bud mites 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 the three orchards (Table 1).

Table 1. Location and virus presence indication for raspberry 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−

RLBV: raspberry leaf blotch emaravirus. Signs (+) and (−) stand for RLBV-infected and RLBV-free raspberry samples, respectively.

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 the orchards (Table 2). The data were 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 the three raspberry orchards were adequately taken in order to obtain representative samples and were further analyzed. Soil pH was measured in a 1M KCl solution by the standard ISS method SRPS EN ISO 10390 [18]. The content of humic substances in soil samples was determined by the standard method ISO 12782-4 [19], while nitrogen was measured by the Kjeldahl method [20]. The determination of the total carbonates, expressed as %$CaCO_3$, 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 [21]. 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) [22].

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 the reverse-transcription polymerase chain reaction (RTPCR). A modified CTAB procedure was used for total nucleic acid (TNA) extraction, as described by Li et al. [23]. For RLBV detection, two-step RT was conducted with random hexamer primers [pd(N)6] and Maxima Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). PCRs were conducted with the RLBV-specific primer pair 1287/1095 that amplified 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

Liquid nitrogen was poured onto the raspberry samples (~150 g), and the frozen fruits were ground in a stainless-steel blender. A powdered sample (10 g) was mixed with 50 mL of 96% ethanol and ultrasonicated at 20 °C. After 30 min of extraction, the mixture was centrifuged two sequential times for 15 min at 960 g (Beckman J21C, Beckman Coulter, Inc., Brea, CA, USA), and the supernatant was filtered through a 0.45 mm Minisart filter (Sartorius, Göttingen, Germany) 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 the total phenolic content [24] and individual polyphenolic profiles. In order to obtain an extract for the anthocyanin content, the identical extraction procedure was repeated but with 50 mL of 96% ethanol/1.5 M HCl (85:15 v/v) [25]. All determinations were performed in triplicate, and the mean $\pm$ standard deviation was presented.

2.5. HPLC-DAD Analysis

As previously published [26], specific phenolic components were quantified using reversed-phase HPLC analysis. Extracts were analyzed utilizing a HPLC Agilent-1200 series (Agilent Technologies, Santa Clara, CA, USA) with a UV–vis DAD detector for multi-wavelength detection. After injection of 5 µL of sample, the separation was performed in an Agilent-Eclipse XDB C-18 column (4.6 $\times$ 150 mm) set at 25 °C. The two elution solvents used were H_2O + 2% formic acid (A) and 80% acetonitrile + 2% formic acid + H_2O (B). The following elution program was utilized: 0–10 min 0% B, 10–28 min gradually increased to 0–25% B, 28–30 min 25% B, 30–35 min gradually increased to 25–50% B, 35–40 min gradually increased to 50–80% B, and 40–45 min gradually decreased to 80–0% B. 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. Standards were purchased from LGC Standards (Teddington, UK).

2.6. Statistical Analysis

A three-factorial experimental design using ANOVA and Tukey's multiple comparison tests was 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 the samples with leaf blotch symptoms (60 samples, 20 per orchard) and confirmed that RLBV was not detected in all the 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 the 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).

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
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, and contents of CY3-GLU, CY3-SOP, PHENOL, and ANTHO are given in mg/100 g fw. Different letters in the same row denote statistically significant difference (Tukey's test, $p < 0.05$) among raspberry fruits of different viral status/locality/year combinations.

All these compounds were previously detected in raspberry fruits of various cultivars [1,27,28]. Fotirić Akšić et al. carried out a comprehensive analysis of the chemical composition of 18 raspberry samples grown in Norway during a two-year period [29]. The authors detected 13 micro- and macronutrients, 11 fruit acids, 22 sugars, and 17 phenolic compounds (anthocyanins were not analyzed). Except ferulic acid, all the other phenolic compounds detected in our raspberry samples were also identified in those samples. The contents of CA, *p*COU, and Q were in good agreement with our results, while the contents of EA, RUT, ISO-Q, Q3-RHA, and KAE were slightly lower, which may be attributed to the specific agroclimatic conditions in Norway. As for the detected and quantified anthocyanins, our results were in great agreement with previously published studies showing that CYA-SOP and CYA-GLU are among the most dominant pigments in raspberry fruits [16,30]. Ponder and Hallmann examined four raspberry cultivars and detected all phenolic acids that were identified in our 'Willamette' raspberry samples. The quantified amount of CA (0.20–0.77 mg/100 g fw), *p*COU (0.63–2.81 mg/100 g fw), FA (0.20–0.95 mg/100 g fw), and EA (36.4–69.3 mg/100 g fw) perfectly matched with our results (3.8–11.9 mg/kg fw for CA, 1.7–26.3 mg/kg fw for *p*COU, 4.9–17.5 mg/kg fw for FA, and 106.3–502.4 mg/kg fw for EA) [31]. In the same study, the obtained results for the detected RUT, Q, and KAE aligned with our own results.

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 the raspberry fruit samples. The factors of variation (viral status, locality, harvest year) and their interactions are presented in Table 4. It is noteworthy that the influence of the harvest year and locality might be actually understood as the influence of weather conditions and soil composition, respectively. As presented in Table 2, there were quite some differences in the average air temperature and average rainfall among the years and localities. On the other hand, the soil composition analysis among the localities (Table 5) showed huge differences, especially in the accessible phosphorus and potassium contents.

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

Viral infection showed the least effect on the contents of CA, Q3-RHA, Q, PHENOL, and ANTHO (no statistical differences), while such an 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 the detected compounds were highly influenced by the harvest year ($p < 0.001$ for all compounds, except *p*COU and Q3-RHA, which were both $p < 0.01$) and locality ($p < 0.001$) (Tables 4 and S1).

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

Locality of Orchard	pH (in 1 M 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

Comparing all the 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 the 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).

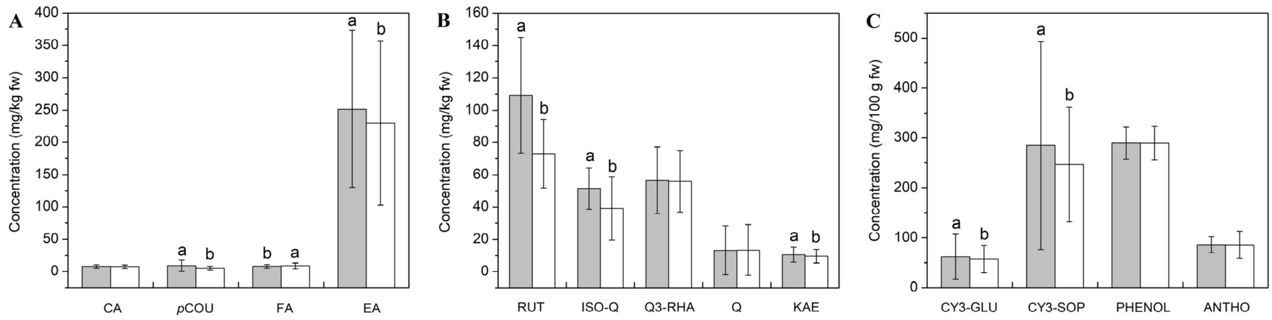

Figure 1. Influence of RLBV on the phenolic profile: (**A**) phenolic acids, (**B**) flavonols, and (**C**) individual and total anthocyanins and total phenolics. For each compound or class of compounds, different letters indicate significant differences ($p < 0.05$) between RLBV-infected and uninfected 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 the infected and healthy raspberry samples of the 'Willamette' in different orchards were grouped based on the concentrations of 11 individual compounds and two classes of compounds (Figure 2). The first two principal components explain 60.02% (39.03% and 20.99%, respectively) of the total variance. The PCA analysis revealed no segregation whatsoever regarding the viral status of the plants. Furthermore, it is evident that infected and uninfected pairs (rounded in Figure 2) stood in close proximity.

The distributions of the 12 raspberry samples along PC1 was mainly related to the concentrations of several compounds among the 11 analyzed individual compounds and two groups of compounds, which showed significant differences in the ANOVA analysis (Table 3). High concentrations of ISO-Q and EA were associated with the raspberry fruits that originated from the localities Cerova and Devići, harvesting in the second (2020) year, regardless of viral status. Higher concentrations of CY3-SOP, CY3-GLU, CA, ANTHO, PHENOL, and FA were associated with the raspberry fruits that originated from all three localities, harvesting in the first (2019) year, regardless of viral status. PC2 separated the raspberry samples mainly based on the contents of Q and Q3-RHA. In other words, the fruits that originated from Bedina Varoš versus the fruits that originated from the other two localities (Cerova and Devići) can be separated along PC2, regardless of the harvesting year and viral status.

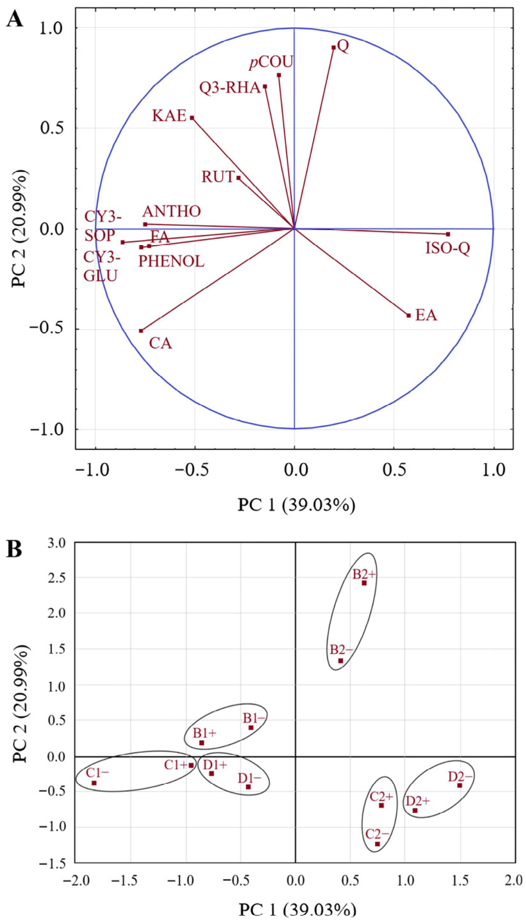

Figure 2. Segregation of 12 raspberry samples according to their polyphenolic profile (11 individual compounds and two groups of compounds) determined by principal component analysis (PCA). (**A**) Variable loadings; (**B**) sample scores. For abbreviations, see the Section 2.

The influence of the harvest year can be more clearly observed in the viral status × harvest year (A × B) and harvest year × locality (B × C) interactions (Table S1). Analyzing the B × C interaction revealed that within the same locality, the content of EA was higher in 2020 compared to 2019, while the content of FA was decreased in 2020. 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, the contents of CY3-GLU in the 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. The interaction A × B showed a very similar trend within the same viral status, meaning that the weather conditions highly affected the formation of certain polyphenolics. The influence of the weather conditions observed through the harvest years can be more clearly seen in the PCA. All the raspberry samples harvested during 2019 were grouped on the negative side of PC1, while the 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 the examined years, the rainfall amounts significantly differed in almost all the examined months (April–October). The locality of Bedina Varoš showed quite some differences regarding the rainfall among the two examined years, especially in July (107.2 and 17.4 mm), September (2.4 and 49.6 mm), and October (5.4 and 152.6 mm). Such a huge discrepancy in monthly rainfall between 2019 and 2020 was noticed only in the Cerova locality in April (101.9 and 22.0 mm, respectively) and in the Devići locality in August (9.3 and 265.0 mm, respectively). In both years, Bedina Varoš was quite rainier compared to the other two localities. The total rainfall (April–October) in Bedina Varoš in 2019 and 2020 was 791.6 and 764.4 mm, in Devići, it was 447.0 and 695.7 mm, while in Cerova, it was 592.6 and 571.8 mm, respectively. As for the average air temperature, one can notice that the Devići locality was slightly colder on a monthly basis during the entire period (April–October) of both the examined years than the Bedina Varoš and Cerova localities. Cheng et al. [32] 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. [33] showed that the contents of almost all anthocyanins were increased in rain-shelter-cultivated wine grapes.

The effect of the locality on the polyphenolic profile cannot be clearly seen through the dual interactions viral status × locality (A × C) and harvest year × locality (B × C). It turned out that the PCA showed good separation among the raspberry samples (Figure 2). Namely, the different behaviors of the individual polyphenolics in the Bedina Varoš locality compared to the Devići and Cerova orchards can be clearly observed in the PCA results. The raspberry samples harvested in the 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 the three orchards (Table 5). Namely, it is clear that the contents of phosphorus and potassium in the soil of the Bedina Varoš orchard were significantly higher compared to the Devići and Cerova orchards, which certainly influenced the polyphenolic profile of the grown fruits [34]. The contents of phosphorus were 27.7-fold and 5.6-fold higher in Bedina Varoš orchard than in the 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 the raspberry fruits appeared to be more influenced by the weather and soil characteristics than by RLBV infection. Given the wide range in the soil composition, particularly in terms of the phosphorus and potassium content, and the 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), this is not surprising. Delgado et al. [35] demonstrated that the anthocyanin content in 'Tempranillo' grapes at veraison increased in proportion to the potassium dose applied in fertilization. Also, an increased accumulation of polyphenols in grape fruits with no nitrogen fertilization was observed, while this trend diminished as the potassium fertilization dose was increased. On the contrary, purple-blue potatoes showed the highest anthocyanin content with the highest nitrogen fertilization and a lack of phosphorus and potassium fertilization [36]. It seems that every fruit and vegetables species and cultivar requires a certain level and ratio of nitrogen, phosphorus, and potassium contents in the soil to maximize the polyphenolic content.

Polyphenolic compounds belong to the group of secondary metabolites responsible for defense mechanisms in plants, primarily towards microbiological infections, including viral infections. The contents of these compounds in fruits depend on various factors, including the fruit species, variety, maturity stage, soil substrate, plant health status, and agricultural practices [37–39]. In this research, the influence of the cultivar was excluded, since only the 'Willamette' cultivar was utilized for the 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 conclusions or trends regarding the influence of the virus presence. The statistical analyses showed an evident influence of the locality and harvest year, since the PCA clearly differentiated all the samples harvested in the first harvest year from the samples harvested the following year, as well as all the samples harvested in the Bedina Varoš locality from all the samples harvested in the remaining two orchards (Figure 2).

It is well known that stress conditions, such as the presence of pathogens, might cause alterations in the contents of flavonoids, anthocyanins, and hydroxycinnamic acids as a sign of a plant's response to infection [40–42]. Such an influence was most obvious in the Bedina

Varoš locality, where all four hydroxycinnamic acids, as well as RUT and ISO-Q, were additionally deposited in the fruits as a consequence of infection during both harvest years (Table 3). Some authors have claimed that an increased amount of hydroxycinnamic acids is a sign of a plant's response to pathogens [43,44]. On the other hand, Usenik et al. [40] found a lower content of hydroxycinnamic acids in plums infected by plum pox virus (PPV) compared to healthy samples. Such an effect was explained by a stress-induced alteration in the biosynthetic pathway of flavonoids, and, thus, an increased amount of flavonoids was synthesized on account of the reduced synthesis of hydroxycinnamic acids in the infected plum samples. In our group's research on PPV's 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 deemed as highly tolerant towards the virus [26]. Tolerance is described as a cultivar's ability to result in a reduced yield or quality loss as a result of disease severity or pathogen development as compared to other cultivars or crops [45]. Agriculture specialists define it as minimal symptom development or a reduction in plant vigor or production in a cultivar despite a normal viral accumulation that would be expected in a sensitive cultivar [46]. In this research, it cannot be concluded that the RLBV infection caused great changes in the examined phenolic profile in the raspberry 'Willamette' samples, but it is also unclear the effect that the virus caused. The effect of the RLBV was indisputable (Tables 3 and 4), but this viral effect on the 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.

The viral infection had no impact on the CA, Q3-RHA, Q, PHENOL, and ANTHO contents (non-significant) and a medium impact on the FA ($p < 0.05$), KAE, and CY3-GLU contents ($p < 0.01$), while a high influence on the *p*COU, EA, ISO-Q, and CY3-SOP contents was detected ($p < 0.001$) (Table 4, Figure 1). Taking this into consideration, it could be concluded that the virus presence definitely showed a certain influence on the polyphenolic compound contents. Nevertheless, such an influence was certainly limited and prevailed due to the effects of the locality and weather conditions. Since these two parameters played an important role, the different behaviors of some phenolic compounds in the two successive harvest years might be explained by the various weather conditions (for instance, the content of CY3-SOP in the 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 the contents of some compounds showed different trends in the various orchards during the same harvest year, such behavior could be due to the different localities and also the different weather conditions (for instance, the content of CY3-GLU in the raspberry samples harvested in Bedina Varoš in 2019 increased as a result of the infection from 69.9 to 132.8 mg/100 g fw, while during the same harvest year, the content of this anthocyanin remained intact after infection in the other two localities). Apparently, the influence of the viral status was overpowered by the influence of the weather and locality on the phenolic profile of the raspberry 'Willamette' samples. 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 orchards' 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 the environmental conditions, which was 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' fruits cannot be indisputably considered invulnerable to RLBV infection regarding the examined phenolic profile. 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 an influence will certainly prevail over the effect of RLBV 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. During the two years of our examination, these two influences overpowered the RLBV infection effect on the chemical composition of 'Willamette' fruits and covered it, as clearly shown by PCA. Such information is of great importance to 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: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10020187/s1, Table S1: Influence of the main variables and their interaction on polyphenolic profile of raspberry fruits.

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