

Impact of raspberry leaf blotch emaravirus on red raspberry ‘Willamette’ fruits

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Received: 8 December 2021

Accepted: 27 December 2021

SUMMARY

Raspberry leaf blotch emaravirus (RLBV) has become established in many Serbian raspberry orchards as the most prevalent virus of raspberries in the country. The aim of this study was to evaluate the impact of RLBV on the red raspberry ‘Willamette’ variety. A trial was conducted in four raspberry orchards located in Western Serbia. Fruits from RLBV-infected and uninfected canes were analyzed for fruit size (fruit length, width, height, shape, and weight), soluble solids content, pH, titratable acidity, total sugars, and total phenolic and anthocyanin contents. The results of the study confirmed that RLBV significantly decreases fruit size and weight (9.15-27.49%) of ‘Willamette’ fruits. Soluble solids content was higher in infected fruits (1.55-7.39%), but the increase was not significant. RLBV did not cause significant changes in titratable acidity of raspberry juice, pH or total sugars content. Total phenolic and anthocyanin contents were higher in fruits of RLBV-infected plants in two out of four locations.

Keywords: raspberry, plant viruses, raspberry leaf blotch emaravirus, raspberry fruits

INTRODUCTION

Raspberries are hosts of more than 40 viruses and virus-like agents. The majority of viruses infecting raspberries do not cause any visible symptoms on leaves or fruits, but some induce different symptoms on infected plants. These symptoms may be easily confused with those caused by other pathogens (mainly fungi) and insects. Raspberry leaf blotch of raspberries has been known for decades and it is manifested by yellow and light green leaf blotches and patches, distortion of leaf margins and twisting of leaves. For a long time, these symptoms were mainly attributed to feeding damage caused by raspberry leaf and

bud mite (*Phyllocoptes gracillis* Nalepa) (Dobrivojević & Petanović, 1985). In the past decade, raspberry leaf blotch disorder has become an important problem for raspberry growing in many countries (Bi et al., 2012; McGavin et al., 2012; Milenković & Marčić, 2011). The development and application of molecular techniques has led to the discovery of a new virus tentatively named raspberry leaf blotch emaravirus (RLBV) (McGavin et al., 2012). The virus was detected in raspberry plants originating from Great Britain and Serbia with leaf blotch symptoms, and in raspberry leaf and bud mites. The RLBV is a member of the genus *Emaravirus*, with segmented, linear, single-stranded negative-strand RNA genome (Elbeaino

et al., 2018). Raspberry leaf blotch emaravirus has been reported to infect numerous raspberry cultivars in several European countries: Bulgaria, Bosnia and Herzegovina, Finland, Great Britain, Montenegro, Poland, Serbia, Slovakia and Ukraine (Delić et al., 2020; Jevremović et al., 2019; Pozhylov et al., 2021). So far, RLBV has not been reported outside Europe. Raspberry leaf and bud mite is a suspected vector of the RLBV.

Red raspberry (*Rubus idaeus* L.) is the most important small fruit in the agricultural production of the Republic of Serbia. Frozen raspberries are one of the most important export products, ranking Serbia one of the world's leading exporters of this commodity (Petrović et al., 2017). Red raspberries are cultivated on more than 14,000 hectares and the main cultivated variety is 'Willamette' with a production share of about 90%. Red raspberry 'Willamette' variety had been bred in Oregon, the United States. It is a mid-summer florican variety with medium-sized fruits. Since the mid-1970s, 'Willamette' has been predominant in raspberry production in Serbia due to its excellent fruit characteristics and suitability of fruits for deep freezing. Raspberries are grown in Serbia according to integrated pest management principles, and in small limited areas in organic farming. Over the last decade, one of the major concerns in raspberry orchard management has been the leaf blotch disorder. Recent studies have confirmed high incidence of RLBV in the country, causing more or less severe symptoms on infected plants (Jevremović et al., 2016, 2019). Also, the raspberry leaf and bud mite, which is a suspected RLBV vector, is considered to be the most important secondary pest of raspberry in Serbia (Milenković & Marčić, 2011).

Since information on the impact of RLBV on infected raspberry plants has been scarce, the aim of our study was to evaluate the influence of RLBV on physical properties and chemical composition of 'Willamette' raspberry fruits in Serbia.

MATERIAL AND METHODS

Experimental design

The trial was conducted during 2019 in four raspberry 'Willamette' orchards located in Western Serbia: Cerova (43° 44.662'N 20° 6.937'E, 336 m altitude), Tvrdići (43° 51.716'N, 19° 56.167'E, 473 m), Bedina Varoš (43° 33.776'N 20° 14.313'E, 686 m), and Deviči (43° 25.667'N 20° 22.999'E, 942 m). Raspberries were trained in all orchards in the linear system with 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. All orchards were maintained according to an integrated pest and disease management system. The raspberry leaf and bud mite was controlled using acaricides according to an insect and disease spray schedule.

Twenty floricanes with leaf blotch symptoms and 20 asymptomatic floricanes were randomly selected from each orchard. In total, 160 canes were selected from 4 orchards. Symptomatic canes expressed typical leaf blotch symptoms: yellow blotches and patches, leaf twisting and distortion (Figure 1). To analyze the presence of RLBV in the selected canes, 5 leaves were sampled from each cane in May 2019. Leaves originating from the same cane represented one sample.



Figure 1. Symptoms of raspberry leaf blotch emaravirus on 'Willamette' leaves

Total nucleic acid extraction and reverse-transcription polymerase chain reaction (RT-PCR) analysis

All collected leaf samples (80 symptomatic and 80 asymptomatic) were analyzed by reverse-transcription polymerase chain reaction (RT-PCR).

A modified CTAB procedure was used for the extraction of TNA (Li et al., 2008). In brief, 200 mg of leaf tissue was ground in 2 ml of 2% cetyltrimethylammonium-bromide (CTAB) buffer in extraction bags (Flexo duga, Serbia). One ml of plant extract was incubated at 65 °C for 15 min and centrifuged at 10,400 rpm for 5 min. Upper aqueous phase (650 µl) was mixed with equal volume of 24:1 chloroform/isoamyl alcohol and centrifuged at 12,800 rpm for 10 min. The supernatant (500 µl) was mixed with 350 µl of ice-cold isopropanol and centrifuged at 12,800 rpm for 10 min. The obtained TNA pellet was washed with 1 ml ice-cold 70% ethanol by centrifugation at 12,800 rpm for 5 min. The pellet was then dried at room temperature and dissolved in 100 µl Tris-EDTA (TE) buffer. The extracted TNA was kept at -20 °C until further analysis.

Two-step reverse transcription (RT) was done with random hexamers [d(N)₆] and Maxima Reverse Transcriptase according to the manufacturer's instruction (Thermo Scientific, USA). PCR reactions were performed with primer pair 1287/1095, which amplifies the 567 base-pair (bp) fragment of the nucleocapsid of RLBV RNA3 (McGavin et al., 2012). Reactions were carried out in a T-personal thermal cycler (Biometra, Germany). Amplified products were analyzed by horizontal electrophoresis in 1.5% agarose gel. After electrophoresis, gel was stained with ethidium-bromide. Visualization of fragments was done in Gel Doc EZ System (Biorad, USA) using UV tray and the presence of the expected fragment of 567 bp was considered as a positive reaction.

To avoid any interaction with RLBV, all samples were also tested to other viruses that are present in Serbia and reported to infect raspberries. Samples were analyzed on the presence of raspberry bushy dwarf virus (RBDV) with ELISA test with reagents of BIOREBA AG, Switzerland. The presence of black raspberry necrosis virus (BRNV), raspberry leaf mottle virus (RLMV), raspberry vein chlorosis virus (RVCV), and rubus yellow net virus (RYNV) was assessed by RT-PCR and PCR, respectively.

Physical properties of fruits

In each orchard/location, fruits from the selected RLBV-infected and RLBV-free canes were hand-picked at the optimal maturity stage during mid-harvest.

Fruits were hand-picked from 20 RLBV-infected and 20 RLBV-free canes (five fruits per cane, respectively) in each orchard, chilled in a field refrigerator and transported to the laboratory. In total, 800 fruits were picked and measured. Fruit dimensions (fruit length, width and height) were measured with digital calipers (Carl Roth, Germany). Fruit shape index was expressed as the ratio of mean height to mean width. Fruit weight was measured with a digital balance XL-1810 (Denver instruments, USA).

Chemical properties fruits

For these measurements fruits were picked from the same canes and on the same date as for the evaluation of fruit physical properties.

Soluble solids content (SSC %, °Brix) in fruits was determined in a small sample of fruit juice with a digital brix MA871 refractometer (Milwaukee, USA) at 20 °C. SCC was determined in 25 individual fruits in each variant. Distilled water was used to calibrate the refractometer.

Fruit pH was measured with a pH meter (Mettler Toledo, Switzerland). Titratable acidity (TA) was determined by neutralization of fruit extract with 0.1 N NaOH at pH 8.2, where phenolphthalein was used as an indicator. The results were expressed as grams (g) of citric acid equivalent per 100 g of fresh weight (FW).

The Luff-Schoorl method was used to evaluate total sugars (TS) (Tanner & Brunner, 1979). The results were expressed in percent per 100 g of FW.

Total anthocyanin content (TAC) was determined following a pH-differential method described by Liu et al. (2002). Absorption was measured at 520 and 700 nm in a spectrophotometer (Beckman, USA). The data are expressed as milligrams of cyanidin-3-glucoside equivalents (C3G) per 100 g FW using a molar extinction coefficient of 26,900.

Total phenolic content (TPC) was determined according to a modified Folin-Ciocalteu colorimetric method (Singleton et al., 1999; Liu et al., 2002). TPC was expressed in milligrams of gallic acid equivalents (GAE) in 100 g FW.

Statistical analysis

The obtained data was analyzed by a one-way analysis of variance (ANOVA) test followed by Duncan's test, where significant differences among the tested traits were assessed at $p < 0.01$ using the CoStat software, version 6.311 (CoHort Software, Monterey, CA, USA).

RESULTS AND DISCUSSION

RT-PCR analysis

All of the 160 collected raspberry leaf samples (40 samples per orchard) were tested for RLBV presence by RT-PCR using the recommended primer pair targeting RNA3 of the RLBV genome (Dong et al., 2016; Jevremović et al., 2019). RT-PCR analysis confirmed RLBV presence in all 80 analyzed samples showing leaf blotch symptoms (20 samples per orchard). RLBV was not detected in any of the 80 analyzed asymptomatic samples (20 samples per orchard). Other viruses (RBDV, RYNV, RLMV, RVCV and BRNV) were not detected in the analyzed samples.

RLBV is a common pathogen in red raspberry orchards in Serbia (Jevremović et al., 2016, 2019). The majority of cultivated raspberry varieties express typical and clear symptoms of RLBV infection on leaves. 'Willamette' has been the prevalent raspberry variety grown in Serbia for decades. Typical leaf blotch symptoms may be observed on infected 'Willamette' plants, as well as some other varieties ('Fertödi Zamos', 'Glen Ample', 'Meeker', 'Polana', and 'Tulameen') reported as RLBV hosts in Serbia.

Physical properties of fruits

The results of our study clearly showed an influence of raspberry leaf blotch emaravirus on fruit dimensions (fruit length, width and height) and weight of infected plants, compared to those from RLBV-free plants (Table 1). The impact of the virus on these traits was confirmed in all four locations.

Depending on location, the decrease in fruit length was 4.01-9.8%, while fruit width decreased 4.06-9.47%, and fruit height 5.88-14.9%. The highest RLBV influence on all examined fruit dimensions was detected in the location Cerova. There were no differences between infected and uninfected fruits regarding the fruit shape index in any location. During this study we did not observe any fruit deformities of fruits from RLBV-infected canes.

The results of statistical evaluation of the tested parameters using variance analysis (ANOVA) showed that RLBV had significant effects on the fruit length, width, height and weight of 'Willamette' raspberry fruits from all four locations ($p < 0.01$) (Table 1). Statistical significance at this level was not achieved only for fruit weight in the location Bedina Varoš, but the difference between mean values of fruit height was found to be significant at $p < 0.05$.

Decrease in fruit weight due to RLBV infection ranged from 9.15-27.49%. As for fruit dimensions, the highest percentage of fruit weight decrease was noted on the location Cerova. Earlier visual field observations had indicated that the fruits on canes with blotch symptoms were generally smaller in size compared to those on canes without any leaf symptoms (Jevremović et al., 2016).

According to reports in literature, the RLBV causes significant production losses to raspberry growers, but no further details are available (Bi et al., 2012; Dong et al., 2016). Our results clearly demonstrated a decrease in fruit weight of the infected 'Willamette' raspberry fruit. The revealed decrease in yield from the test orchards is directly proportional to the percentage of infected canes. Differences between orchards regarding fruit weight decrease were evidenced. They can be attributed to factors such as the specific environmental conditions existing in different locations and the applied agro-technical measures.

There are no other literature data on the influence of RLBV on red raspberry fruit. The presence of the virus has been confirmed in several European countries with more or less significant raspberry fruit production. The variety structure differs from country to country and the influence of RLBV should be studied in other important cultivars.

Fruit chemical properties

The obtained values of SCC, TA, pH and TS are presented in Table 2. Soluble solids contents were higher in RLBV-infected canes on all four locations. The increase

Table 1. The effect of raspberry leaf blotch emaravirus (RLBV) infection on fruit length, width, height, fruit shape index and weight of 'Willamette' raspberry

Evaluated trait	Location							
	Tvrđići		Bedina Varoš		Devići		Cerova	
	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+
Fruit length (mm)	19.43 ± 0.35 a	18.65 ± 0.23 b	19.12 ± 0.44 a	18.14 ± 0.48 b	20.62 ± 0.81 a	18.86 ± 0.44 b	19.79 ± 0.54 a	17.83 ± 0.48 b
Fruit width (mm)	18.19 ± 0.30 a	17.46 ± 0.21 b	18.09 ± 0.31 a	16.97 ± 0.50 b	18.38 ± 0.47 a	17.12 ± 0.52 b	18.19 ± 0.46 a	16.60 ± 0.39 b
Fruit height (mm)	21.45 ± 0.73 a	19.83 ± 0.42 b	19.54 ± 0.37 a	18.39 ± 0.67 a	22.26 ± 0.83 a	20.05 ± 0.73 b	21.43 ± 0.32 a	18.22 ± 0.52 b
Fruit shape index	1.07 ± 0.01 a	1.07 ± 0.01 a	1.06 ± 0.01 a	1.07 ± 0.01 a	1.12 ± 0.02 a	1.10 ± 0.02 a	1.09 ± 0.02 a	1.07 ± 0.03 a
Fruit weight (g)	3.28 ± 0.18 a	2.98 ± 0.18 b	2.95 ± 0.13 a	2.52 ± 0.19 b	3.85 ± 0.24 a	2.92 ± 0.24 b	3.31 ± 0.15 a	2.40 ± 0.14 b

Letters differing row-wise per location indicate significant difference ($p < 0.01$)

in SCC ranged from 1.55-7.39%. The highest SCC increase was evidenced on the location Cerova (7.39%). Statistical evaluation of SCC mean values showed that RLBV had no significant impact on this trait ($p < 0.01$) in fruits from any of the examined localities.

There was no statistically significant effect of RLBV on titratable acidity on three out of four locations. The obtained TA values of raspberry juice made from infected fruits were up to 11.04% higher compared to uninfected fruits within each locality.

The determined pH values of fruits from RLBV-infected plants were equal or up to 1.93% lower than those from uninfected plants. No statistically significant effect of RLBV was found at $p < 0.01$, except on the location Cerova.

Total sugars content in infected fruits was higher on locations Tvrdíci and Cerova (by 4.05 and 15%, respectively), but lower in Bedina Varoš and Devíci

(by 4.84 and 8.21%, respectively). There were no significant differences between RLBV-infected and -uninfected plants regarding total sugars within each examined location.

The RLBV did not have a crucial role in changes of total sugars content in the infected fruits. Statistical analysis showed that RLBV had no effect on this trait at $p < 0.01$.

Total anthocyanin content in RLBV-infected and -uninfected raspberries from four locations is presented in Figure 2. A large variation was observed among values of total anthocyanin content for 'Willamette' raspberry from different locations, regardless of RLBV infection. In two locations (Bedina Varoš and Cerova) TAC values were higher in fruits from infected plants. On the other two localities (Devíci and Tvrdíci), TAC values were lower in fruits from infected plants. Total phenolic content was shown to correlate with total anthocyanin content (Figure 2).

Table 2. Soluble solids content, titratable acidity, pH value of the fruit juice, and total sugars content in fruits from RLBV-infected (RLBV+) and RLBV-uninfected (RLBV-) plants

Evaluated trait	Location							
	Tvrdíci		Bedina Varoš		Devíci		Cerova	
	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+	RLBV-	RLBV+
Soluble solids content (% °Brix)	9.48 ± 1.37 a	9.82 ± 1.54 a	8.39 ± 1.02 a	8.52 ± 0.57 a	8.67 ± 0.87 a	9.09 ± 1.02 a	10.28 ± 0.81 a	11.04 ± 0.82 a
Titratable acidity (% w/v, citric acid)	1.63 ± 0.03 a	1.65 ± 0.04 a	1.33 ± 0.18 a	1.71 ± 0.05 b	1.66 ± 0.07 a	1.69 ± 0.05 a	1.83 ± 0.10 a	1.87 ± 0.19 a
pH	3.27 ± 0.05 a	3.27 ± 0.04 a	3.17 ± 0.07 a	3.15 ± 0.07 a	3.07 ± 0.05 a	3.05 ± 0.00 a	3.11 ± 0.01 a	3.05 ± 0.01 b
Total sugars	4.26 ± 0.18 a	4.44 ± 0.12 a	3.72 ± 0.36 a	3.54 ± 0.06 a	4.38 ± 0.30 a	4.02 ± 0.06 a	5.10 ± 0.18 a	6.00 ± 0.36 a

Letters differing row-wise per location indicate significant difference ($p < 0.01$)

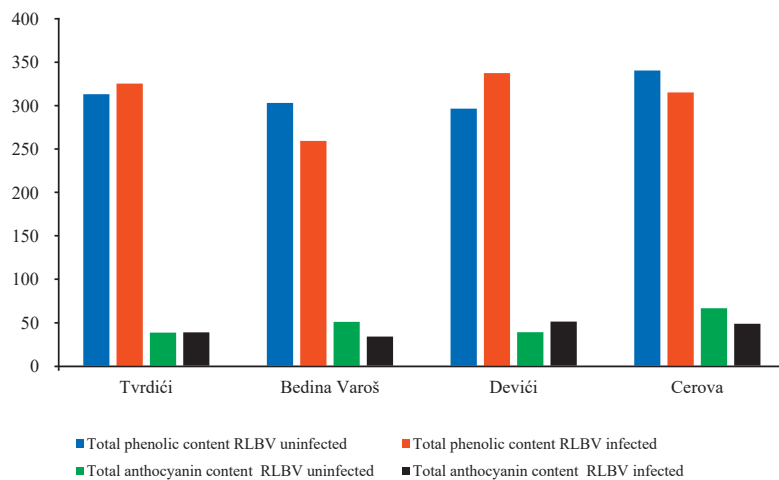


Figure 2. Total phenolic and total anthocyanin contents in raspberry fruits from RLBV-infected and RLBV-uninfected plants

Anthocyanins are an important class of flavonoids that represent a large group of plant secondary metabolites. Environmental growing conditions can affect the ability of fruit species to synthesize anthocyanin (Howard et al., 2003). Other important factors that influence total anthocyanins content are: fruit species, variety, maturity stage, soil substrate, and plant health status. Kalt et al. (2001) also reported that the synthesis of anthocyanins and phenolic compounds can be influenced by biotic and abiotic factors (irradiation, temperature, and pathogen attacks). The only factor that varied among plants in the examined locations was RLBV infection. RLBV infection increased total anthocyanin and total phenolic contents in some locations, while decreasing them in others.

CONCLUSIONS

The presented results clearly demonstrate that the RLBV significantly decreased the dimensions and weight of infected raspberry ‘Willamette’ fruits. Soluble solids content was higher in fruits collected from RLBV-infected plants, but with no significant effect. The analysis confirmed that RLBV did not have a statistically significant impact on pH, titratable acidity or total sugars content in fruits from infected plants. Total anthocyanin and total phenolic contents were higher in fruits from RLBV-infected plants on two examined locations, but lower on the other two localities.

ACKNOWLEDGEMENTS

This work was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, contract 451-03-68/2022-14/200215.

The authors would also like to express gratitude to Ivan Radojčić, BSc in agronomy, and students of the Čačak Faculty of Agronomy Miodrag Gizdavić, Dragomir Marković and Stefan Popović for their assistance during field activities.

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Utica j virusa mrljavosti lista maline na plodove maline sorte 'Willamette'

REZIME

Virus mrljavosti lista maline (raspberry leaf blotch emaravirus, RLBV) je prisutan u velikom broju zasada maline u Srbiji i najrasprostranjeniji je virus maline u zemlji. Cilj ovog rada je bio ispitivanje uticaja RLBV na plodove maline sorte 'Willamette'. Ogladi su sprovedeni u četiri zasada maline u lokalitetima zapadne Srbije. Analizirani su plodovi sa RLBV zaraženih i nezaraženih izdanaka: dužina, širina i visina ploda, indeks oblika ploda, težina ploda, sadržaj rastvorljive suve materije, pH vrednost voćnog soka, titraciona kiselost, ukupni šećeri, sadržaj ukupnih fenola i antocijana. Rezultati istraživanja su pokazali da je virus mrljavosti lista maline imao značajan uticaj na smanjenje veličine i težine (9,15-27,49%) ploda maline. Sadržaj rastvorljive suve materije je bio viši kod zaraženih plodova (1,55-7,39%), ali ovo povećanje nije bilo od statističkog značaja. RLBV nije imao uticaj na titracionu kiselost voćnog soka, pH vrednost i sadržaj ukupnih šećera. Sadržaj ukupnih fenola i antocijana je bio viši kod plodova sa zaraženih izdanaka u dva od četiri ispitivana lokaliteta.

Ključne reči: malina, biljni virusi, virus mrljavosti lista maline, plodovi maline

