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# *Raspberry bushy dwarf virus* – a Grapevine Pathogen in Serbia

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**SUMMARY**

During a field survey in 2005 in vineyards and grapevine nurseries at localities in central Serbia, a few plants with unusual virus-like symptoms were observed. Leaf samples were analyzed by DAS-ELISA for the presence of nine viruses. Besides other viruses frequently occurring on grapevine, *Raspberry bushy dwarf virus* (RBDV) was detected in two samples. Results were confirmed by nested-PCR and sequence analysis of the fragment in 5' part of RNA-1. Obtained sequence shared at least 93% of nucleotide identity with the compared RBDV sequence originating from raspberry. The finding of *Raspberry bushy dwarf virus* on grapevine in Serbia is a second finding of this pathogen on grapevine worldwide. The first natural infection of grapevine with this virus was reported in Slovenia in 2003.

**Keywords:** *Raspberry bushy dwarf virus*; Grapevine; Detection; DAS-ELISA; Nested-PCR

## INTRODUCTION

*Raspberry bushy dwarf virus* (RBDV), genus *Idaeovirus*, is a seed- and pollen-borne virus that is commonly found in red and black raspberry. It seems to occur worldwide. Natural hosts belong to the genus *Rubus*. In most raspberry cultivars (*R. idaeus*) infection with this virus is symptomless, but in sensitive ones, the virus can cause leaf curling, necrosis, premature defoliation, decreased vigour, and drupelet abortion leading to crumbly fruit. In loganberry *(R. loganobaccus*), boysenberry (*R. ursinus*) and black raspberry (*R. occidentalis*) symptoms are uncertain. Although the virus causes mild symptoms, it may affect fruit quality, especially in mixed infections. Experimentally, RBDV was transmitted to more than 50 herbaceous hosts.

RBDV genome is segmented, bipartite and consists of three segments of linear, positive-sense, single-stranded RNA. RNA-1 contains a large ORF that encodes the replicase, which is used to copy the viral RNA to make complementary RNA, which serves as a template for the new viral RNA. The 5' part of RNA2 encodes the viral movement protein (MP) which facilitates cell to cell movement of the virus. RNA3, which is derived from the 3' part of RNA2, encodes the coat protein (CP) which makes the protein shell that protects the viral RNA.

*Raspberry bushy dwarf virus* is present and described in Serbia on two red raspberry varieties. In early 90's,

a resistant-breaking strain was described on Willamette variety (Dulić-Marković and Ranković, 1992). In Meeker variety, RBDV was detected for the first time in 2004 (Jevremović et al., 2004). Willamette, which is predominant and represents 90% of total raspberry production in Serbia, is resistant. On Meeker variety, which is described as sensitive one, RBDV may induce interveinal chlorosis and leaf yellows, as well as crumbly fruits.

In 2003, RBDV was reported for the first time to naturally infect grapevine (*Vitis vinifera*), a host outside the genus *Rubus* (Mavrič et al., 2003). Further investigations confirmed that RBDV is widespread in Slovenia in numerous, mostly white grapevine varieties (Mavrič Pleško et al., 2009).

In this paper the results of serological and molecular detection of *Raspberry bushy dwarf virus* on grapevine in Serbia are presented.

## MATERIAL AND METHODS

### Material

During 2005, in a field survey in vineyards and grapevine nurseries in the region of Trstenik and Kruševac unusual leaf symptoms on 3 plants of the unknown white vine variety at the locality Velika Drenova were observed. These symptoms suggested the virus nature of the disease.

### DAS-ELISA test

Leaf samples were tested for the presence of nine viruses: *Grapevine virus* A (GVA), *Grapevine leafroll associated virus* 3 (GLRaV-3), *Grapevine fleck virus* (GFkV), *Arabis mosaic virus* (ArMV), *Raspberry bushy dwarf virus* (RBDV), *Raspberry ringspot virus* (RpRSV), *Tomato black ring virus* (TBRV), *Tomato ringspot virus* (ToRSV) and *Strawberry latent ringspot virus* (SLRSV). Analysis was done by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) with the reagents of BIOREBA AG, Switzerland according to the manufacturer's recommendation. Leaf tissue was extracted in extraction buffer (TRIS-Tween + 2% PVP + 1% PEG) in 1/20 (v/w) ratio. The color development was measured at 405 nm with an ELISA reader (MULTISKAN MCC/340) after 30-120 min. Samples were considered positive if the OD values were two times higher than the OD values of the negative control.

### Nested-PCR

For the confirmation of RBDV presence in two ELISA-positive grapevine samples, nested-PCR method was performed. RNA extraction from the 100 mg of leaf samples was done with the RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. For nested-PCR, two sets of RBDV specific primers were selected (Barbara et al., 2001). The first PCR was done with N3 (5'-GGT GTC TGG CTG TTT AAG CG-3') and N4 (5'-GGG ATA TAG CCA CTT GTA GCG-3') primer set. PCR conditions for the reaction were as follows: reverse transcription and denaturation 45 min at 42°C, 2 min at 92°C/ 20 cycles of 92°C-1 min, 56°C-1 min, 72°C-1 min; 72°C-5 min. Subsequently, one µl of reaction was transferred to second PCR with primers 268 (5'-ACG GTG CTA TTA TGA CTG TGT-3') and 269 (5'-GGT AGG TCA TAA CCG GAA TG-3'). PCR conditions for second PCR reaction were as follows: 2 min at 92°C; 35 cycles of 92°C-1 min, 56°C-1 min, 72°C-1 min; 72°C-5 min. Amplified products were analyzed on 5% polyacrylamide gel stained with silver-nitrate.

### Sequencing

Selected amplification products with 268/269 primer set were purified and sequenced (MACROGEN, South Korea). Nucleotide sequence of the isolate named VD was deposited in the GenBank under accession number JF262960 (http://www.ncbi.nlm.nih.gov/). Nucleotide sequence was compared with the sequence from red raspberry isolate R15 from Great Britain (acc. number S51557) using BioEdit software (Hall, 1999).

## RESULTS AND DISCUSSION

DAS-ELISA test confirmed that all three samples were infected with more than one virus. Symptoms of the ELISA-positive samples were a yellow mosaic pattern, bright yellow bands along the main veins, curved line pattern and partial chlorosis of leaves (Figures 1-3).

One sample was infected with *Grapevine leafroll associated virus* 3. In the second sample *Grapevine fleck virus* and *Raspberry bushy dwarf virus* were found. The third sample, with the most unusual symptoms, was infected with four different viruses: *Grapevine virus* A, *Grapevine fleck virus*, *Arabis mosaic virus* and *Raspberry bushy dwarf virus.* DAS-ELISA test results are shown in table 1.

**Figure 1.** Vein clearing and yellow patterns

**Figure 2.** Mosaic pattern

**Figure 3.** Irregular line pattern

**Table 1.** OD values ($A_{405}$) measured after 60 min at room temperature (RT)

| | Viruses | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample nr. | GVA | GLRaV-3 | GFkV | ArMV | RBDV | RpRSV | TBRV | ToRSV | SLRSV |
| 1 | 0.195 | **0.586*** | 0.118 | 0.171 | 0.175 | 0.115 | 0.102 | 0.105 | 0.107 |
| 2 | 0.026 | 0.124 | **0.905** | 0.250 | **0.794** | 0.126 | 0.109 | 0.126 | 0.123 |
| 3 | **0.716** | 0.144 | **1.197** | **0.438** | **0.688** | 0.115 | 0.103 | 0.119 | 0.132 |
| -[1] | 0.166 | 0.117 | 0.119 | 0.185 | 0.150 | 0.120 | 0.108 | 0.117 | 0.125 |
| +[2] | **2.801** | **1.246** | **0.852** | **1.988** | **1.237** | **0.897** | **0.571** | **0.625** | **0.663** |

* - Positive values are shown in bold; [1]–negative control; [2]–positive control

*Grapevine virus* A, *Grapevine leafroll associated virus*, *Grapevine leafroll associated virus* 3, *Grapevine fleck virus* and *Arabis mosaic virus* were already described on grapevines in Serbia (Kuzmanović et al., 2003). Besides mentioned viruses, Paunović et al. (2007) described *Grapevine leafroll associated virus* 2 and *Grapevine fan leaf virus,* but for none of them a linear mosaic is a characteristic symptom. *Grapevine virus* A does not induce

specific symptoms on foliage. *Grapevine leafroll associated virus* induces downward rolling and discoloration of the leaves, which turn reddish or yellowish, depending on the variety. Infection with *Grapevine fleck virus* in European varieties is symptomless. In susceptible varieties, *Arabis mosaic virus* induces leaf mottling and flecking.

To confirm ELISA-positive results, nested-PCR method with RBDV specific primers was used. The results of nested-PCR test were in complete agreement with DAS-ELISA test results. PCR products of 1062 bp in size were produced from both samples using RBDV specific primers.

In order to confirm the finding of a new pathogen on grapevine in Serbia, the fragment of 941bp located in 5' terminal part of RNA-1 of RBDV genome of two isolates was sequenced. Sequence alignment based on nucleotide (nt) sequence in this region was used for reliable identification of RBDV. Both analyzed samples had identical sequence and resembled the same isolate, named VD. Our isolate was compared with the only available nt sequence for this region /R15 isolate from raspberry/ (Ziegler et al., 1992) and shared 93.62% of nt sequence identity. Nucleotide sequence of Serbian RBDV isolate from grapevine in comparison with R15 isolate is shown in Figure 4. Serbian isolate has 3 nucleotide insertions ATA at the position 899-901.


**Figure 4.** Nucleotide sequence alignment of Serbian RBDV isolate VD from grapevine and R15 isolate from raspberry. Dots (.) show identity with nt sequence of VD isolate; nucleotide insertions are located at the positions 899-901.

*Raspberry bushy dwarf virus* is a common virus on raspberry. It is present in Serbia in Willamette and Meeker varieties (Dulić-Marković and Ranković, 1992; Jevremović et al., 2004). Depending on the variety, it can cause losses in yield and fruit quality. With the domination of Willamette variety in the production, reported incidence of RBDV was low and did not represent a significant threat to raspberry production in Serbia (Jevremović et al., 2009).

At present, more than 40 viruses from different families and genera are reported on grapevine. Some of these

viruses, such as *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Broad bean wilt virus* (BBWV), *Potato X virus* (PVX), *Artichoke Italian latent virus* (AILV) and *Alfalfa mosaic virus* (AMV) are economically important pathogens for many cultivated crops (Martelli and Walter, 1999). For grapevine, they represent scientific curiosity, because they are rarely found or cause no damage. The occurrence of RBDV on grapevine is curiosity and it was found for the first time in Slovenia (Mavrič et al., 2003). RBDV isolates are widespread in many grapevine varieties in Slovenian vineyards, but its epidemiology and economic importance are not documented.

The presence of RBDV on grapevine in Serbia is the second finding worldwide. Extensive survey, through inspection and subsequent testing is needed to determine whether this is a single event or RBDV is more spread within grapevine such as in Slovenia.

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# Virus žbunaste kržljavosti maline (*Raspberry bushy dwarf virus*) – patogen vinove loze u Srbiji

**REZIME**

Pregledom vinograda i rastila vinove loze tokom 2005. godine u lokalitetima centralne Srbije na nekoliko biljaka su primećeni neuobičajeni simptomi koji su ukazivali na moguće prisustvo virusa. Uzorci listova su analizirani DAS-ELISA testom na prisustvo 9 virusa. Pored virusa koji se često javljaju na vinovoj lozi, kod dva uzorka je potvrđeno prisustvo virusa žbunaste kržljavosti maline (*Raspberry bushy dwarf virus*, RBDV). Nested-PCR metodom i sekvencionom analizom fragmenta iz 5' regiona RNA-1 segmenta genoma RBDV potvrđeni su rezultati seroloških testa. Dobijena nukleotidna sekvenca pokazuje najmanje 93% sličnosti sa upoređenom sekvencom RBDV izolata iz maline. Potvrda prisustva RBDV na vinovoj lozi u Srbiji je drugi nalaz ovog patogena na vinovoj lozi u svetu. Prvi nalaz prirodne infekcije vinove loze ovim virusom saopšten je u Sloveniji 2003. godine.

**Ključne reči:** Virus žbunaste kržljavosti maline; vinova loza; detekcija; DAS-ELISA; nested-PCR