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Original scientific paper

Incidence of viruses in highbush blueberry (*Vaccinium corymbosum* L.) in Serbia

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SUMMARY

A large-scale survey for highbush blueberry (*Vaccinium corymbosum* L.) viruses in Serbia was performed from 2011 to 2015. A total of 81 leaf samples from 15 locations were collected and analyzed for the presence of 8 viruses. Serological ELISA assay was performed to determine the presence of: *Blueberry scorch virus* (BlScV), *Blueberry shock virus* (BlShV), *Blueberry shoestring virus* (BSSV), *Blueberry leaf mottle virus* (BLMoV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV). All samples were tested for the presence of *Blueberry red ringspot virus* (BRRV) by PCR and for *Blueberry mosaic-associated virus* (BlMaV) by RT-PCR test. The analyses confirmed the presence of BlMaV in 8 (9.9%) samples and BRRV in 1 (1.2%) sample. No BlScV, BlShV, BLMoV, BSSV, TRSV or ToRSV viruses were detected in any of the analyzed samples.

Keywords: Blueberries; *Vaccinium*; Plant viruses; Serbia

INTRODUCTION

The highbush blueberry (*Vaccinium corymbosum* L.) is considered one of the most commercially important berry crops. Blueberry is an antioxidant-rich fruit species and recent findings of its nutritional value have resulted in a constant increase in its production. The major world producers are the United States, Canada and Chile (Leposavić, 2014). Highbush blueberry was introduced into Serbia 40 years ago, but a significant increase in its production started over the past decade. In 2005, highbush blueberry was cultivated on no more than 5 ha in Serbia, but the area increased to approximately 220 ha at the end of 2015. All highbush blueberry plantations in Serbia were established with the imported planting material, mostly from Germany, Poland and the Netherlands.

Highbush blueberry hosts a variety of viruses and virus-like agents (Martin et al., 2012). Economically the most important viruses of blueberries are: *Blueberry red ringspot virus* (BRRV), *Blueberry leaf mottle virus* (BLMoV), *Blueberry scorch virus* (BlScV), *Blueberry shock virus* (BlShV), *Blueberry shoestring virus* (BSSV), *Peach rosette mosaic virus* (PRMV), *Tobacco ringspot virus* (TRSV) and *Tomato ringspot virus* (ToRSV) (Prodorutti et al., 2007). *Blueberry latent virus* (BBLV), *Blueberry latent spherical virus* (BLSV), *Blueberry virus A* (BVA) and *Blueberry mosaic-associated virus* (BlMaV) have been recently reported as new viruses in blueberry (Isogai et al., 2011; Martin et al., 2011; Isogai et al., 2013; Thekke-Veetil et al., 2014). In sensitive blueberry cultivars, viruses may cause great yield losses, reduce fruit quality or destroy entire bushes. Conversely, infection may remain latent in tolerant cultivars and such plants may serve as a reservoir of inoculum. Viruses spread via infected plant material and vectors, such as nematodes and insects. The basic

preventive measures for virus disease control include the use of virus-free planting material and spatial isolation from other blueberry plantations. Prompt elimination and removal of symptomatic or diseased plants may not give satisfactory results because of the long incubation period of some viruses.

Research of blueberry viruses in Serbia have been initiated recently as the fruit growing area increased (Paunović et al., 2011). In this paper, we present the results of the first large-scale survey for virus presence in highbush blueberry plantations in Serbia.

MATERIAL AND METHODS

Plant material

Selected highbush blueberry plantations on 15 locations in Serbia were visually inspected from 2011 to 2015 (Figure 1). Plantation age ranged from 3 to more than 30 years. A total of 81 samples from symptomatic and asymptomatic bushes were collected. Each sample consisted of 8-10 randomly selected leaves from one plant. The material was kept at +4°C for serological and/or at –20°C for molecular tests.

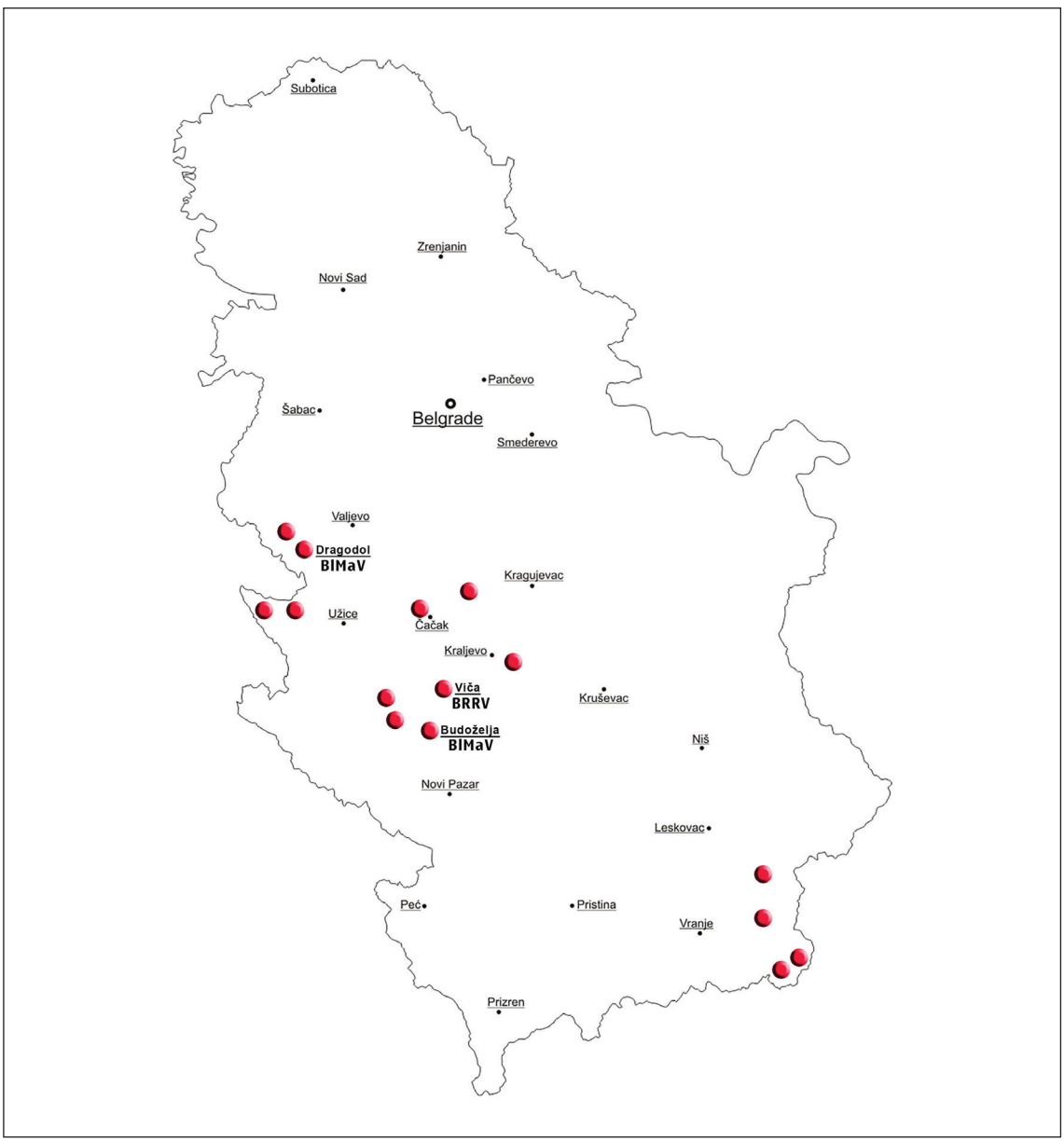

Figure 1. Map of Serbia showing sampling locations (indicated by red dots) and viruses detected in highbush blueberry plantations

Serological tests

All collected samples were serologically tested for the presence of six viruses: *Blueberry leaf mottle virus, Blueberry scorch virus, Blueberry shock virus, Blueberry shoestring virus, Tobacco ringspot virus* and *Tomato ringspot virus.* Assays were performed by enzyme-linked immunosorbent assay (ELISA) with the reagents of AGDIA Inc. (USA) for the detection of BLMoV, BlScV, BlShV and BSSV; and BIOREBA AG (Switzerland) for the detection of TRSV and ToRSV, according to the manufacturers' recommendations. Fresh leaf samples were prepared at 1:10 ratio in the general extraction buffer (GEB) (AGDIA Inc.). Color development was measured at 405 nm for BLMoV, BlScV, BlShV, TRSV and ToRSV; and 650 nm for BSSV on ELISA reader (MULTISKAN MCC/340) after 20-120 min. Samples were considered as positive when optical density (OD) values were at least three times higher than the OD values of the negative control.

Molecular tests

Total nucleic acids (TNA) were extracted from fresh or frozen leaves with a modified CTAB method as described in detail by Li et al. (2008). The extracts were stored at –20°C before use. The samples were analyzed for the presence of *Blueberry mosaic-associated virus* by reverse transcription – polymerase chain reaction (RT-PCR). The first-strand cDNAs were generated by reverse transcription reactions using Maxima Reverse Transcriptase (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The obtained cDNAs were used as templates in PCR reactions with specific BlMaV primers that amplify a fragment from the 3' part of RNA 1 of the BlMaV genome (Thekke-Veetil et al., 2014). The PCR conditions were: 94°C for 3 min for initial denaturation; followed by 40 cycles at 94°C for 20 s, 50°C for 20 s and 72°C for 1 min; and final extension at 72°C for 7 min. For the detection of *Blueberry red ringspot virus,* a primer pair RR-13/RR-14 was used for PCR amplification of the fragment located in the CP gene of the BRRV genome (Glasheen et al., 2002). The program for PCR was: 94°C for 5 min, followed by 35 cycles at 94°C for 40 s, 50°C for 40 s and 72°C for 60 s, followed by a final extension step at 72°C for 5 min. TPersonal thermocycler (Biometra GmbH, Germany) was used in these PCR reactions. PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by Gel Doc EZ System (Biorad laboratories, USA).

RESULTS

During inspection, a majority of plants in the surveyed plantations did not show any symptoms indicating virus presence. Symptoms typical for blueberry mosaic disease were observed on the leaves of 7 plants on 2 locations. The plants showed yellow, red, yellowish green or white mosaic pattern on leaves (Figures 2 and 3). No other symptoms on stem or fruits were found on these plants. One plant showed reddish blotches on stem indicating the presence of BRRV (Figure 4).

Figure 2. Mosaic symptoms on blueberry leaves – light green mosaic pattern induced by *Blueberry mosaic-associated virus*

Figure 3. Mosaic symptoms on blueberry leaves – yellowish and dark red mosaic pattern induced by *Blueberry mosaic-associated virus*

Figure 4. Reddish blotches on blueberry stem caused by *Blueberry red ringspot virus*

The results of the ELISA tests on the presence on BLMoV, BlScV, BlShV, BSSV, TRSV and ToRSV revealed that none of the 81 analyzed leaf samples were positive. The OD values of all samples for each tested virus were at the level of negative control. A positive result was obtained only in the positive control for each tested virus, with OD values at least 3 times higher than the value for negative control.

The RT-PCR analysis on BlMaV presence revealed an expected 756 bp PCR fragment in 8 analyzed samples (Figure 5). BlMaV was detected in all 7 samples of 'Bluecrop' and 'Duke' blueberry cultivars with mosaic symptoms. The virus was also detected in one asymptomatic sample of cv. 'Duke'. The asymptomatic plant was located just next to two BlMaV infected plants with symptoms of yellow and light green mottle and mosaic pattern. BlMaV was detected in 3 symptomatic samples from a 30-years-old plantation on the location Dragodol; and in 5 plants from a 10-years-old plantation on the location Budoželja (Figure 1).

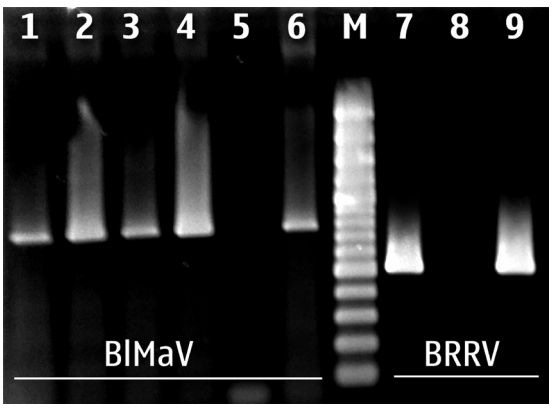

Figure 5. Detection of BlMaV and BRRV in blueberry samples. Lanes: 1 - isolate 36, 2 - isolate 37, 3 - isolate 38, 4 - isolate 39, 5 - BlMaV negative control, 6 - BlMaV positive control, M - GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, USA), 7 - isolate 20, 8 - BRRV negative control, 9 - BRRV positive control.

BRRV infection was confirmed in 1 sample of blueberry 'Bluetta' showing reddish blotches on stem. A PCR fragment of 487 bp was obtained from the sample collected from a 15-years-old plant on the location Vič̆a (Figure 5).

DISCUSSION

Most of the analyzed samples were negative on the presence of any of the analyzed viruses. This could be attributed to the virus-free planting material and spatial remoteness of new plantations from the old ones. The most frequent virus detected in this study was BlMaV, confirmed in 9.9% of the samples. Blueberry mosaic disease (BMD) has been known for more than 60 years, but the causative agent was described only recently (Thekke-Veetil et al., 2014). The effect of the disease on fruits and yield has not yet been fully investigated. In some symptomatic bushes, fruits ripen late, and their quality and yield are lower (Ramsdell & Stretch, 1987). BMD has been described in many countries, but so far the presence of BlMaV has been confirmed in the USA, Slovenia, Turkey and Japan (Thekke-Veetil et al., 2014; Gauthier et al., 2015; Thekke-Veetil et al., 2015; Gazel et al., 2015; Isogai et al., 2015). The presence of BlMaV was also recently reported in Serbia (Jevremović et al., 2015). Our survey confirmed a localized occurrence of BlMaV in Serbia on two remote plantations (approximately 100 km). RT-PCR analysis was proved to be a reliable diagnostic tool for the detection of BlMaV in symptomatic and asymptomatic samples, as reported by Thekke-Veetil et al. (2014). The detection of BlMaV in an asymptomatic plant confirmed a latent infection and indicated a need for inspection of imported material, i.e. plants from nurseries and field, in order to control the pathogen. Soil-borne fungi of the genus *Olpidium* are the suspected vectors of BlMaV (Thekke-Veetil et al., 2014).

BRRV was recently reported on 2 highbush blueberry plants in Serbia (Jevremovic et al., 2014), on a location that recurred in this present study. *Blueberry red ringspot virus* was first reported in the United States and later on in Japan, Czech Republic, Slovenia, Poland and Korea (Cho et al., 2012; Mavrič Pleško et al., 2010). A 25% reduction in yield of the infected blueberry 'Blueray' was reported in a study in the United States (Martin et al., 2012). The natural spread of BRRV in the field was evidenced and a mealybug was suspected to be a vector.

In our study, no other viruses were detected in the analyzed samples. Some of the viruses that infect blueberry have been reported in several European countries. *Blueberry leaf mottle virus* was originally reported as *Grapevine Bulgarian latent virus* in Bulgaria, Hungary and Portugal, but it was later shown to be a distinct species, not a separate strain (EPPO, 1997; Elbeaino et al., 2011). *Blueberry scorch virus* was reported in Italy, Poland and the Netherlands (Ciuffo et al., 2005; Paduch-Cichal et al., 2011). *Blueberry shoestring virus*, *Peach rosette mosaic virus* and *Tobacco ringspot virus* were detected in Poland (Paduch-Cichal et al., 2011). BLMoV, BlScV, TRSV and ToRSV were put on the EPPO A2 List of pests recommended for regulation as quarantine pests (EPPO, 2015). BLMoV, TRSV and ToRSV are on the list of harmful organisms not known to occur in the territory of the Republic of Serbia but whose introduction

to and spread within the Republic of Serbia has already been banned in List IA part I (Anonymous, 2012). Imported planting material is regularly inspected by an authorized laboratory at the Fruit Research Institute, Čačak, for the presence of BLMoV, BlScV, TRSV and ToRSV. During inspection, no positive samples have been detected (unpublished data).

This study is the first large-scale survey of viruses in highbush blueberry plantations in Serbia. To date, only a localized occurrence of BlMaV and BRRV has been confirmed. Dissemination of these viruses from their first detected site to other locations has not been evidenced. Imports of plant material from countries with reported viruses infecting blueberry involve a risk of introducing these pathogens. With a steady increase in area under highbush blueberry cultivation in Serbia, we may expect to detect other viral diseases as well. The application of proper detection methods is of great importance for early identification of viruses and reducing their dispersion to disease-free areas. All infected bushes should be removed and destroyed. Other important control approaches include the use of certified planting material, spatial isolation of new from old plantations and regular vector control.

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Prisustvo virusa američke visokožbunaste borovnice (*Vaccinium corymbosum* L.) u Srbiji

REZIME

Ispitivanje prisustva virusa američke visokožbunaste borovnice (*Vaccinium corymbosum* L.) u Srbiji sprovedeno je u periodu 2011–2015. godine. Sakupljen je i analiziran 81 uzorak lišća borovnice iz 15 lokaliteta na prisustvo 8 virusa. Serološki ELISA test je primenjen za ispitivanje uzoraka na prisustvo: virusa sprženosti borovnice (*Blueberry scorch virus* – BlScV), virusa šoka borovnice (*Blueberry shock virus* – BlShV), virusa nitavosti lišća borovnice (*Blueberry shoestring virus* – BSSV), virusa šarenila lista borovnice (*Blueberry leaf mottle virus* – BLMoV), virusa prstenaste pegavosti duvana (*Tobacco ringspot virus* – TRSV) i virusa prstenaste pegavosti paradajza (*Tomato ringspot virus* – ToRSV). Na prisustvo virusa crvene prstenaste pegavosti borovnice (*Blueberry red ringspot virus* – BRRV) uzorci su analizirani PCR metodom, a na prisustvo virusa mozaika borovnice (*Blueberry mosaic-associated virus* – BlMaV) RT-PCR metodom. Rezultati ispitivanja su potvrdili prisustvo BlMaV u 8 (9,9%) i BRRV u 1 (1,2%) uzorku. Prisustvo BlScV, BlShV, BLMoV, BSSV, TRSV i ToRSV nije dokazano ni u jednom ispitivanom uzorku.

Ključne reči: Borovnica; *Vaccinium*; Biljni virusi; Srbija