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Review paper

Plum pox virus strains: Diversity and geographical distribution in Serbia

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SUMMARY

Plum pox virus (PPV) is the causal agent of Sharka disease. Since its discovery, Sharka has been considered as a calamity in plum orchards. PPV is present worldwide in many *Prunus* species, causing great economic losses. In highly susceptible plum varieties, such as Požegača, PPV causes a premature fruit drop and reduces fruit quality, which leads to total yield loss. Eight PPV strains (PPV-M, PPV-D, PPV-EA, PPV-C, PPV-Rec, PPV-W, PPV-T and PPV-CR) have been recognized so far. Three major strains (PPV-M, PPV-D and PPV-Rec) are the most widely dispersed and occur frequently in many European countries. Other strains are of minor importance due to their limited host preferences or geographic distribution. So far, all three major strains have been identified in Serbia. In this paper, we provide a comprehensive overview of the research into *Plum pox virus* variability in Serbia.

Keywords: *Plum pox virus*; Sharka disease; Variability; Serbia

INTRODUCTION

Plum pox virus (PPV) belongs to a group of 'Top 10' plant viruses in molecular plant pathology based on their perceived scientific or economic importance (Scholthof et al., 2011). PPV causes Sharka, the most deleterious viral disease of stone fruits that infects many commercial, ornamental and wild species in the genus *Prunus*. Sharka disease was first described on plum in Bulgaria by Atanasov (1932) and since then it has spread to many countries across Europe, Asia, North Africa, South and North America (Barba et al., 2011).

Plum pox virus is a member of the genus *Potyvirus* in the *Potyviridae* family (King et al., 2012). Genome organization of PPV is typical of *Potyviruses* and contains one large Open Reading Frame (ORF) expressed as a polyprotein precursor of 355.5 kDa. The PPV polyprotein is co- and post-translationally cleaved by three virus-encoded proteinases to produce 10 protein products: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa, VPg, NIb and CP (Salvador et al., 2006). Another short ORF, called PIPO (Pretty Interesting Potyviridae ORF), has been reported for *Potyviruses* recently (Chung et al., 2008). PIPO is embedded within the P3 cistron and translated as a fusion protein in the +2 reading frame. The encoded protein is a fusion with the N-terminal part of P3, giving rise to P3N-PIPO.

PPV infects many important cultivated species of the genus *Prunus*: European plum (*Prunus domestica* L.), Japanese plum (*P. salicina* Lindl.), apricot (*P. armeniaca* L.), peach (*P. persica* (L.) Batsch), sweet cherry (*P. avium* L.), sour cherry (*P. cerasus* L.) and almond (*P. amygdalus* L.).

Figure 1. Chlorotic rings, and patterns on plum leaves caused by *Plum pox virus*

Figure 2. Požegača fruits deformed by *Plum pox virus* infection

Figure 3. Severe Sharka symptoms on peach leaves

Figure 4. Deformed and bumpy apricot fruits induced by *Plum pox virus*

Infected *Prunus* species growing along roads, in hedges and in urban areas are also PPV hosts and usually serve as reservoirs of infection, such as Myrobalan plum (*P. cerasifera* Ehrh.) and blackthorn (*P. spinosa* L.). The first herbaceous host of PPV, *Nicotiana quadrivalvis* Pursh., was reported by Sutic (1961). More than 70 herbaceous species from 9 families may be naturally or artificially infected with PPV (Llácer, 2006).

Plum pox virus causes symptoms on leaves, fruits, flowers, branches and seeds (Figures 1-4). Depending on the sensitivity of any particular variety, symptoms may be less or more severe.

The primary way of PPV long distance dispersal is through movement of infected plant material. Once introduced into a new environment, PPV is efficiently vectored by leaf aphids in a non-persistent manner. More than 20 species have been reported as PPV vectors, but the most efficient are: *Aphis craccivora* Koch, *Brachicaudus helicrysi* Kalt*, B. cardui* L.*, Myzus persicae* Sulz and *Phorodon humuli* Schr (Labonne et al., 1995; Kegler & Hartmann, 1998). PPV is not a seed-borne virus.

Plum pox virus strain variability

The first attempts to detect PPV and characterize strains had been based on the response of herbaceous and woody indicator plants. The first studies had focused on determining the most reliable indicator plants. The European plum cvs Požegača and Crvena Ranka were the first plants used for PPV diagnosis. Požegača showed very high susceptibility but long incubation period and nonuniformity of its seedlings made it an unsuitable indicator (Ranković, 1975). Crvena Ranka seedlings have proved to be reliable indicators in many experiments conducted by Jordović (1957) and Ranković (1975). Seedlings of *Prunus tomentosa*, proposed as PPV indicators by Jordović (1961), have proved to be reliable PPV indicators (Ranković, 1975, 1980). PPV experiments on peach seedlings as test plants were first reported by Šutić (1963). At the same time, many herbaceous plants were tested as PPV indicators. Németh (1963) discovered *Chenopodium foetidum* as a reliable test plant, and Kassanis and Šutić (1965) described *Nicotiana clevelandii* as a useful plant for indexing plums and other fruit trees. Many other herbaceous plants have also been described as more or less reliable PPV indicator plants (Németh, 1986). Today, PPV indexing on herbaceous plants is restricted to *N. benthamiana* because of the lacking specificity and reliability of most other herbaceous hosts (Llácer, 2006).

The first clear division of PPV strains was shown by Šutić et al. (1971) describing three types of PPV symptoms on *C. foetidum*: yellow (Y), intermediate or necrotic-yellow (Y/N) and necrotic (N). The yellow strain caused yellow spots on leaves that developed slowly without defoliation. The intermediate strain caused yellow spots with necrosis at the center and some of the leaves dropped off later on. The necrotic strain caused necrotic lesions and falling of the inoculated leaves. This strain characterization was used during the 1970s, even in cases when it was difficult to clearly distinguish the symptoms. All three described strains were efficiently detected using *P. tomentosa* as an indicator plant. Symptoms on *P. tomentosa* could not be used for differentiation of PPV strains but they were useful for differentiation of diseases caused by other viruses, such as *Apple chlorotic leafspot virus*, *Prune dwarf virus* and *Prunus necrotic ringspot virus* (Ranković, 1980). Later, Damsteegt et al. (1997) confirmed that a US hybrid line of *P. tomentosa* was suitable to distinguish PPV-D and PPV-M isolates based on the expressed symptoms. Vineyard peach seedlings had proved to be unreliable indicators of different PPV isolates (Ranković, 1975). Intensive symptoms on vineyard peach leaves were observed only with the N strain, but Y and Y/N isolates produced either no symptoms at all or the symptoms were not clear enough. Currently, the main woody indicator plants recommended for PPV diagnosis are the peach GF305 and *P. tomentosa* (Anonymous, 2004).

The development of serological Enzyme Linked Immunosorbent Assay (ELISA) and its ensuing use in plant virology has improved PPV diagnosing (Adams, 1978). Compared to biological tests, ELISA was recognized as a rapid and sensitive method. The method was immediately thereafter implemented at the Fruit Research Institute in Čačak for PPV detection in different hosts and sample types (Ranković & Vuksanović, 1981, 1985; Paunović et al., 1988). Differentiation of isolates based on limited serological cross-reactions by double immuno-diffusion tests in agar, using polyclonal antibodies and formaldehyde-treated suspensions of purified viral particles, was studied by Kerlan and Dunez (1979). For the first time two different PPV serotypes were described: D (Dideron) and M (Marcus). Based on the mobility of CP (coat protein) in denaturing Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), two groups of isolates with different molecular weights (36k Da and 38 kDa) were discriminated (Adamolle, 1993). These groups corresponded to the PPV-M and PPV-D strains. The correlation between coat protein (CP) mobility and strain affiliation (Y, Y/N and N) of PPV isolates using "disc" electrophoresis were first reported by Ranković and Veličković (1983). In Slovakia, Šubr and Glasa (1999) described the differences between the migration of different strains in SDS-PAGE. The CP of PPV-M migrated faster than the CP of PPV-D, while the CP of PPV-Rec in most cases migrated as a double-band. The double-band pattern was initially attributed to mixed infections or it was considered to be a laboratory artifact. Later, this double-band pattern was shown to be associated with PPV-Rec (Glasa et al., 2005; Subr et al., 2007; Kollerová et al., 2008). Differences in CP mobility between different Serbian PPV isolates/strains have also been reported (Paunović & Jevremović, 2002; Paunović et al., 2006). The production and use of specific monoclonal antibodies improved PPV diagnosis by ELISA technique. Monoclonal strain-specific antibodies (Mabs) specific to structural PPV CP have been developed for: PPV-D (Cambra et al., 1994), PPV-M (Boscia et al., 1997), PPV-EA (Myrta et al., 1998), PPV-C (Myrta et al., 2000) and PPV-W (Croft et al., 2008). A PPV-M specific antibody detects both PPV-M and the later described PPV-Rec isolates and this analysis needs to be supplemented with molecular tests for precise strain identification.

The application of Polymerase Chain Reaction (PCR) for *Plum pox virus* improved the detection and characterization of this pathogen (Wetzel et al., 1991b; Wetzel et al., 1992). The most frequently targeted region in PPV analysis is the highly variable 5'-terminal part of the CP gene. Many strain-specific primers targeting this and other parts of the PPV genome have been developed for single or mixed infection detection (Candresse et al., 1995; Candresse et al., 1998; Nemchinov & Hadidi, 1998; Glasa et al., 2002b; Šubr et al., 2004; Glasa et al., 2005; Glasa et al., 2011; Glasa et al., 2013). PPV strains can also be discriminated by PCR-RFLP analysis, but some isolates may be mistakenly classified. Real-time PCR is an adaptation of the traditional PCR protocol that allows rapid detection of target-specific amplicons without post-PCR electrophoresis. This assay was successfully developed for PPV diagnosis and characterization (Schneider et al., 2004; Olmos et al., 2005; Varga & James, 2005). The use of molecular techniques, availability of full-length and partial sequences and phylogenetic analysis have enabled precise and reliable strain classification.

Eight subgroups of PPV stains have been described so far: PPV-M, PPV-D, PPV-EA, PPV-C, PPV-Rec, PPV-W, PPV-T and PPV-CR (Šubr & Glasa, 2013). PPV-M, PPV-D and PPV-Rec are considered as major strains.

PPV-M

The PPV-M (*Marcus*) strain was initially characterized on a Greece-originating peach (Kerlan & Dunez, 1979) and it infects peach, apricot and plum. The PPV-M strain is present in many countries across East and Central Europe and the Mediterranean and it is efficiently transmitted by aphids, particularly in peach orchards (Dallot et al., 2003, 2004; Capote et al., 2010). Phylogenetic analysis of the collected isolates with different geographical origin has shown that there are two different PPV-M clades, named Ma and Mb (Dallot et al., 2011). The isolates in the Ma clade originated from Mediterranean countries, whereas those of the Mb clade originated from eastern and central European countries. The existence of two subgroups of PPV-M isolates, based on their serological relationship, had already been reported earlier by Myrta et al. (2001).

PPV-D

The PPV-D strain (*Dideron*) was originally described on apricot from France (Kerlan & Dunez, 1979). PPV-D isolates are present in many European countries, South and North America and Asia. The PPV-D strain infects apricot, plum and peach. Compared to PPV-M, PPV-D is described as a less epidemic and nonaggressive strain. This general description is not always correct because strain epidemicity depends on many factors, i.e. the specific reaction of any given isolate, host or aphid species. PPV-D isolates may also induce epidemics in peach, apricot and plum (Gottwald et al., 1995; Dallot et al., 1998; Polák & Komínek, 2009).

PPV-EA

PPV-EA (*El Amar*) was described by sequence analysis of the 3'-terminal part of the genomes of PPV isolates from Egypt (Wetzel et al., 1991a). PPV-EA is present only in Egypt and it is of minor importance. Shalaby et al. (2003) described PPV-EA infection of apricot and its very mild symptoms on plum and peach.

PPV-C

The first finding of PPV on sour cherry was reported in Moldova (Kalashyan et al., 1994). Later, it was also described on sweet and sour cherries in Bulgaria, Croatia, Italy, Hungary, Romania and Belarus (Glasa et al., 2013). As a new strain, PPV-C (*Cherry*) was proposed by Nemchinov et al. (1996). Even though sweet and sour cherries are natural hosts of PPV-C, these isolates may also be potentially transmitted to other *Prunus* species (Bodin et al., 2003).

PPV-Rec

The recombinant PPV isolate ŏ6, originating in Serbia, was first described by Cervera et al. (1993). After another recombinant isolate, BOR-3, was found in Slovakia, new protocols for detection of these isolates were developed (Glasa & Šubr, 2001; Glasa et al., 2002b; Šubr et al., 2004). The high frequency of recombinant PPV isolates had been overlooked for a long time because all typing methods focused on the CP gene or CP protein. PPV-Rec isolates are natural homologous recombinants between PPV-M and PPV-D isolates. The recombination breakpoint is located at the nucleotide position 8450 (based on the full-length nucleotide sequence of the isolate BOR3, GenBank # AY028309). As a new strain, PPV-Rec (*Recombinant*) was proposed by Glasa et al. (2004). Recombinant PPV isolates have a D-type of genome, except that the C-terminal part of NIb gene, CP gene and 3' noncoding region are M-type. PPV-Rec isolates are widespread in many Central and Eastern European countries, as well as in Canada, Pakistan and Turkey. PPV-Rec naturally infects plum and apricot and it is efficiently transmitted by aphids. Before a report by Kamenova et al. (2011) PPV-Rec had been considered unable to infect peach naturally (Glasa et al., 2002a; Glasa et al., 2004; Glasa et al., 2005; Jevremović, 2008).

PPV-W

Based on serological and molecular analysis, James and Varga (2005) described a PPV isolate newly discovered in Canada, named W3174, as a member of a newly proposed PPV-W (*Winona*) strain. PPV-W isolates have been recently discovered in Russia, Ukraine and Latvia (Glasa et al., 2011).

PPV-T

As a novel strain, PPV-T (*Turkey*) was proposed by Serce et al. (2009). PPV-T isolates have been detected on plum and apricot in the region of Ankara, Turkey,

and shared the same recombination event in the HC-Pro gene around the genome position 1566.

PPV-CR

The last reported PPV strain was PPV-CR (Cherry Russia), found naturally infecting sour cherry in the Samara and Saratov regions of Russia, as well as in urban ornamental plantings in Moscow (Chirkov et al., 2013; Glasa et al., 2013).

TYPING OF PPV STRAINS IN SERBIA

After the first report of PPV in Bulgaria, Josifović (1937) carried out the first survey for Sharka (called Plum mosaic at the time) in Serbia. The survey was carried out in many orchards in four counties located close to the border on Bulgaria. Josifović found that many plum trees south of the river Nišava and east of the rivers Južna Morava and Vardar were infected with PPV. Diseased trees were found both in young and old orchards, while the percentage of infected trees ranged from 50-80% in some orchards. In other locations (Belgrade, Užice and Vršac) PPV was detected only on several trees (Pobegajlo, 1940). For a long time, plum and apricot had been the only natural hosts of PPV among cultivated *Prunus* species in Serbia. The first PPV infected peach was discovered in the region of Subotica, close to the Hungarian border in 1984 (Dulić & Šarić, 1986).

The first characterization of PPV strains in Serbia was based on the reaction of *C. foetidum*, as described by Šutić et al. (1971). In various studies, all three known strains (Y, Y/N and N) were detected among the tested Serbian PPV isolates. Analyzing a large number of plum and peach PPV isolates by serological and molecular techniques, Dulic-Markovic (2003) indicated the presence of two strains in Yugoslavia that belonged to the M and D serotypes. A great majority of the isolates belonged to PPV-M (68%), while 13% belonged to PPV-D. A third group of the isolates (19%) from plum and apricot trees in central Serbia (Čačak and Valjevo) were of the M serotype, had a coat protein migration characteristic of M type and tested negative to a panel of D Mabs. However, all of them were found to have the *RsaI* restriction site on a 243 bp amplified fragment, which is typical of D isolates. None of the isolates could infect peach. The existence of PPV-M and PPV-D isolates in Serbian plum and peach samples was also reported in other studies using monoclonal antibodies (Paunović & Jevremović, 2002, 2003; Paunović et al., 2006). The presence of PPV-Rec strain in Serbia was confirmed in several plum samples from central Serbia (Glasa et al., 2005). The identification of PPV-Rec isolates was based on RT-PCR/RFLP tests targeting the CP and P3-6K1 genome parts. As an addition, RT-PCR with subgroup specific primers enabling direct discrimination of PPV-M, PPV-D and PPV-Rec isolates was also done. Subsequent investigation of PPV strains was based on a standardized IC-RT-PCR procedure with strain-specific primers targeting CP and CI genomic regions (Jevremović et al., 2007b; Dallot et al., 2008; Jevremović, 2008, 2013).

The presence of the minor PPV-C strain in Serbia was studied by Paunović and Jevremović (2009). More than 100 leaf samples of sweet and sour cherry from the Fruit Research Institute collection orchard were analyzed. The PPV-C strain was not detected in any of the analyzed samples.

PRESENT STATUS OF PPV STRAINS IN SERBIA

PPV-M

A large-scale analysis of peach, plum, apricot and Myrobalan samples from numerous locations in Serbia has confirmed the presence of the PPV-M strain in all *Prunus* hosts (Jevremović, 2008, 2013). Overall, this strain was present in 23% of the analyzed samples (Jevremović & Paunović, 2013). PPV-M was detected as a largely prevailing strain in peach samples in Serbia, with only several samples affected with PPV-D alone or a mixed infection of PPV-M and PPV-D (Dulic-Markovic, 2003; Dulic-Markovic & Jevremovic, 2006; Dallot et al., 2008; Jevremović, 2008). Shortly after the discovery of PPV in peach in Serbia, the strain rapidly spread into many peach orchards (Dulić et al., 1987). Today, PPV-M is present in almost all inspected locations in Serbia (Figure 5). Geographical distribution of PPV-M is closely associated with peach and it is therefore the prevalent strain in the region of Belgrade and in Vojvodina (Jevremović, 2008). In some other hosts, PPV-M is much sparser, occurring in 8.7% and 27.5% of plum and apricot samples, respectively (Jevremović & Paunović, 2013). As in Serbia, the PPV-M strain has also been found essentially predominant in peach in Bulgaria, the Czech Republic, France, Slovakia and Slovenia (Dallot et al., 2008).

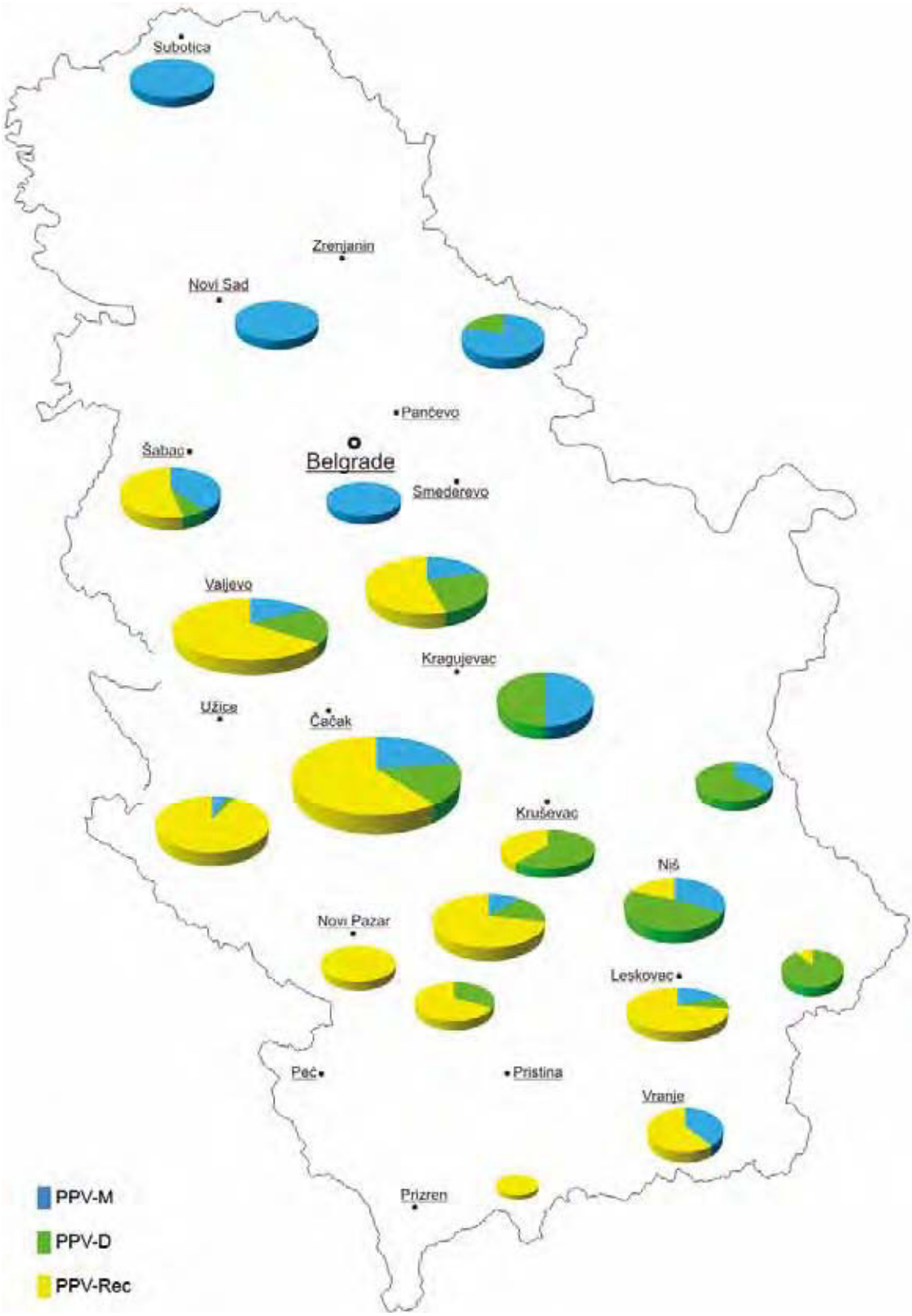

Figure 5. Geographical distribution of three major PPV strains in Serbia

PPV-D

The PPV-D strain has been detected in all tested *Prunus* species in Serbia (i.e. plum, apricot, peach and Myrobalan), accounting for 23% of all detected infections. PPV-D was rarely found in peaches (in several samples only), but much more in apricot and plum, 8.7% and 25.6%, respectively (Jevremović & Paunović, 2013). The PPV-D strain is widespread in all fruit-growing regions in Serbia and prevails in the Nišava, Pirot, Rasina and Zaječar districts (Figure 5). That geographic region was the location where plum mosaic symptoms had been noticed for the first time in Serbia (Josifović, 1937). Based on a high genetic diversity of Serbian PPV-D isolates, as well as the prevalence of that strain in the historic region of Sharka in Serbia, Jevremović (2013) assumed that PPV-D was "the oldest" strain in the country.

PPV-Rec

The first recombinant isolate (ŏ6) among *Potyviruses* was detected in Serbia (Cervera et al., 1993). The authors assumed that the recombination event occurred at low frequency because it requires the joining of two compatible genomic fragments, and virus efficiency in competing with other well-adapted strains. The discovery of several recombinant isolates in Serbia in the early 2000s (Glasa et al., 2005) prompted further investigation into PPV-Rec frequency in different *Prunus* hosts and its geographic distribution. The first survey for PPV-Rec isolates in Serbia showed that the isolates were present on 2/3 of the territory of Serbia (Jevremović et al., 2007a). A further study confirmed that the recombinant strain was the most prevalent strain (43.7%) in Serbia overall (Jevremović & Paunović, 2013). PPV-Rec is the prevalent strain in plum and apricot, found in 53.5% and 52.5% of the analyzed samples, respectively. Not a single peach tree has been found to be infected with recombinant isolates in any survey (Jevremović et al., 2007b; Dallot et al., 2008; Jevremović, 2008). Many reports from studies conducted in the 1980s had described a strain that infected plum and apricot trees, but could not infect peach. Šutić and Ranković (1983) concluded that PPV strains found in plum and apricot samples in Serbia were not adapted to peach. Many peach cultivars have been described as resistant to the PPV strain infecting plum and apricot (Ranković & Šutić, 1980; Jordović, 1985; Ranković & Šutić, 1986). The described strain could only be diagnosed by *P. tomentosa*, and not by vineyard peach or GF 305 seedlings (Ranković, 1975; Jordović, 1985; Ranković & Šutić, 1986). A group of isolates from central Serbia with these characteristics was also described by Dulic-Markovic (2003). The high prevalence of PPV-Rec in Serbia and the fact that it cannot infect peach suggest that the strain described in all those studies is indeed the recombinant strain. Based on a phylogenetic analysis of a limited number of recombinant isolates from Serbia a hypothesis was formulated that PPV-Rec originated in the territory of ex-Yugoslavia (Glasa et al., 2005). PPV-Rec is prevalent in 8 Serbian districts (Jablanica, Kolubara, Kosovska Mitrovica, Mačva, Moravica, Šumadija, Toplica and Zlatibor) (Figure 5) and it is well-adapted to plum and apricot (Jevremović, 2008, 2013). The wide distribution and high prevalence of PPV-Rec suggest that the strain has been present in Serbia for a long period of time.

CONCLUSION

Decades of continuous *Plum pox virus* research in Serbia show that it is the most studied plant virus in the country. The most comprehensive survey of the frequency and geographical distribution of three major PPV strains was carried out in the past decade. Its results confirmed that all major PPV strains in Serbia were present in different *Prunus* hosts. The PPV-Rec strain proved to be the most widespread strain in plum and apricot, but not in peach.

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Sojevi virusa šarke šljive: diverzitet i geografska rasprostranjenost u Srbiji

REZIME

Virus šarke šljive (*Plum pox virus*-PPV) je prouzrokovač bolesti šarke šljive. Od otkrića, šarka je viđena kao bolest od velikog značaja u zasadima šljive. PPV je prisutan širom sveta u mnogim vrstama iz roda *Prunus*, izazivajući velike ekonomske gubitke. Kod veoma osetljivih sorti šljive, kao što je Požegača, virus šarke izaziva prevremeno opadanje polodova i smanjuje njihov kvalitet što dovodi do totalnih gubitaka prinosa. Do sada je opisano osam sojeva virusa šarke: PPV-M, PPV-D, PPV-EA, PPV-C, PPV-Rec, PPV-W, PPV-T i PPV-CR. Tri glavna soja: PPV-M, PPV-D i PPV-Rec su najviše rašireni i prisutni su u mnogim evropskim zemljama. Ostali sojevi su zbog ograničenog broja domaćina ili ograničene geografske rasrostranjenosti od manjeg značaja. U Srbiji su do sada opisana sva tri glavna soja virusa šarke. U ovom radu, dat je sveobuhvatan pregled istraživanja varijabilnosti virusa šarke šljive u Srbiji.

Ključne reči: *Plum pox virus*; virus šarke šljive; varijabilnost; Srbija