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GENETIC DIVERSITY OF *Pseudomonas syringae* PV. *syringae* ISOLATED FROM SWEET CHERRY IN SOUTHERN AND NORTHERN REGIONS IN SERBIA

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Bacterial canker and leaf spot caused by plant pathogenic bacterium *Pseudomonas* is among the most destructive cherry diseases worldwide. Nowadays in Serbia, sweet cherry production significantly increased and the new plantations, mainly grown from imported planting material are being raised every year. During spring, 2018 and 2019, occurrence of bacterial canker and leaf spot symptoms was observed on a newly planted sweet cherry plantations in two localities, Žitorađa (Southern region) and Karavukovo (Northern region-Vojvodina). Typical *P. syringae* colonies were isolated on Nutrient Sucrose Agar supplemented with 5% sucrose (NSA). A total of fifteen isolates were selected and identified. Results of the LOPAT test (+---+) determined them to belong to fluorescent *Pseudomonas* Group Ia, while results of $G^{+}A^{+}T^{-}Ta^{-}$ tests indicate presence of *Pseudomonas syringae* pv. *syringae*. Pathogenicity was confirmed on immature sweet and sour cherry fruitlets by forming of black, sunken lesions for all tested isolates. Genes *syrB* and *syrD* were successfully detected in all tested isolates. DNA sequencing using *gapA*, *gltA*, *gyrB* and *rpoD* housekeeping genes determined tested isolates to belong to *P. s.* pv. *syringae* using the National Center for Biotechnology Information (NCBI) nucleotide BLAST. The Serbian isolates shared 99.47% to 100% (Žitorađa) and 99.38% to 100% (Karavukovo) identity with bacterium *P. s.* pv. *syringae*. Phylogenetic analysis grouped isolates from Žitorađa in one tree cluster, separate from the Karavukovo isolates,

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indicating presence of two genetically diverse groups of causal pathogen *P. s.* pv. *syringae*, obtained from two geographically distinct localities in Serbia. Phylogeographic analysis grouped isolates from Žitorađa in multilocus haplotype coded as REz and isolates originated from Karavukovo in multilocus haplotype coded as REk. Considering that during last few years *P. syringae* continuously occurs mainly in young sweet cherry plantations, where imported material is used for raising, health status check is recommended to be included as obligatory measure when nursery material is used from import.

Keywords: bacterium, bacterial canker, leaf spot, sweet cherry, plantation

INTRODUCTION

Pseudomonas syringae is a plant-associated bacterium widely distributed on over 180 host plants, including different fruit trees, vegetables, field crops and ornamental plants whereby causing various symptoms manifested as: flower blast, spot and blister on fruits, leaf spot, gummosis, shoot dieback and steam canker (AGRIOS, 2005; YOUNG, 2010). Bacterial canker caused by *P. syringae* pathovars (pvs.): *syringae*, *morsprunorum* (race 1 and race 2) and *cerasi* is one of the major disease that affecting sweet cherry production causing significant losses which are expressed through the yield reduction and death of whole trees. Disease is mostly distributed in young sweet cherry plantations worldwide and in Serbian region also (VICENTE *et al*., 2004; GILBERT *et al*., 2010; SPOTTS *et al*., 2010; ILIČIĆ, 2016; BALAŽ *et al*., 2016; ILIČIĆ *et al*., 2018, 2019).

The following symptoms appeared on the sweet cherry plants infected with *P. syringae* pvs.: death buds, branch and stem canker, gummosis and leaf spot with shot-hole appearance. The dormant buds placed on the branches, twigs or stems start with decaying. The bud base first turns brown followed by the whole bud drying, while the necrosis gradually spreads to the woody part. In the commercial orchards, wounds arisen during pruning and heading cuts present another particularly troublesome entry points for the pathogen introduction. Presence of the pathogen leads to rapid decay of young cherry trees (SPOTTS *et al*., 2010; ILIČIĆ, 2016). Leaf spot symptom on sweet cherry occurs sporadically. During the spring and under cool and wet conditions bacteria from the epiphytic population multiply rapidly, causing brown-purple spots that drop out and produce "shot hole" symptoms (KENELLY *et al*., 2007; BULTREYS and KALUZNA, 2010; SPOTTS *et al*., 2010; ILIČIĆ, 2016; ILIČIĆ *et al*., 2018).

Control of the *P. syringae* pvs. is generally very difficult and mainly focused on agrotechnical and phytosanitary measures. Pruning, as one of the main agro-technical measure should be carried out in the early summer with obligatory required tool disinfection. As one of the most important preventative measures are the production and the use of healthy planting material, compatible rootstocks and cultivars and less susceptible cultivars to pathogen. Chemical control, in general, implies preventive use of fungicides based on copper compounds, but there is a restriction for copper usage, in terms that treatments of stone fruits should only be performed during the dormant period when it is safe for the host (KENNELLY *et al*., 2007; CARROLL *et al*., 2010; ILIČIĆ, 2016; ILIČIĆ *et al*., 2018).

During spring, in May 2018 and 2019, bacterial canker symptoms were observed on a newly planted sweet cherry plantations in two Serbian regions (localities Žitorađa - Southern

Serbia and Karavukovo – Northern Serbia, Vojvodina) raised with imported planting material. Therefore the aim of present study was to determine the causal agent of the disease using conventional bacteriological methods and molecular technique polymerase chain reaction (PCR) for preliminary identification and for the purpose of Multilocus Sequence Typing and Analysis (MLST/MLSA) and construction of the haplotype network.

MATERIALS AND METHODS

Isolation and purification of bacterial isolates

During spring (May) in 2018 and 2019, survey was performed on a newly planted sweet cherry plantations in two Serbian localities – Žitorađa (Toplički region) and Karavukovo (Bačka region), respectively. Samples that were consisted of stems and branches with bacterial canker and leaves with leaf spot as disease symptoms were collected. They were first disinfected with 70% ethanol, rinsed under tap water and dried. Small fragments taken from the margins of the healthy and diseased tissues were homogenized in sterile distilled water (SDW) and left for 20 minutes in case of leaves samples or 2 hours in case of woody tissue samples. Obtained suspensions were plated onto the nutrient agar supplemented with 5% (*w/v*) of sucrose (NSA) (LELLIOTT and STEAD, 1987) and incubated at 26 °C for 48-72 hours. After the incubation period, levan - type bacterial colonies, characteristic for the genus *Pseudomonas* fluorescent Group Ia (LELLIOTT *et al*., 1966) were purified and stored at -20 °C in Luria broth (LB medium) with 20% glycerol. A total of fifteen isolates were selected for further work (Table 1).

Table 1. List of the isolates used in this study and NCBI Accession numbers

Strain name	Locality	Year	Symptom	NCBI GenBank Accession numbers			
				gapA	*gltA*	*gyrB*	*rpoD*
RE3	Žitorađa	2018	Bacterial	MT543299	MT543297	MT543295	MT543292
RE31			canker	-	-	-	-
RE32				-	-	-	-
RE33				-	-	-	-
RE34				-	-	-	-
RE4	Žitorađa	2018	Bacterial	MT559826	MT559825	MT543294	MT559824
RE41			leaf spot	-	-	-	-
RE42				-	-	-	-
RE43				-	-	-	-
RE44				-	-	-	-
RE01	Karavukovo	2019	Bacterial	-	-	-	-
RE02			canker	-	-	-	-
RE03				-	-	-	-
RE04				-	-	-	-
RE05				MT543298	MT543296	MT543293	MT543291

In all provided tests, following reference strains of *P. syringae* pvs. were used: *syringae* (strain KFB0103), *morsprunorum* race 1 (strains KFB0120 *cfl* + and KFB0121 *cfl* -) and *morsprunorum*

race 2 (strain KFB0101), all obtained from the Collection of Plant Pathogenic Bacteria, Faculty of Agriculture Belgrade, Serbia.

Pathogenicity

Pathogenicity of fifteen tested isolates was checked on immature sweet and sour cherry fruitlets. They were first washed, dried and then disinfected with 70% of ethanol. Isolates were grown on Nutrient Agar (NA) at 26ºC for 48 hours, after that bacterial cells were suspended in SDW and adjusted to the final concentration of 10^8 CFU mL^{-1} by using McFarland Densitometer DEN-1B (Biosan). Inoculation was performed by the needle to make holes, where the drops in amount of 15 µL were fulfilled (KALUZNA and SOBICZEWSKI, 2009). SDW and reference strains were used as negative and positive controls, respectively. Experiment was performed in three replicates. Inoculated fruitlets were kept in Petri dishes in laboratory conditions, at room temperature and high relative humidity for 3 days.

Biochemical tests

Isolates were examined for biochemical tests such as Gram reaction, presence of catalase, production of fluorescent pigment on King's B medium under UV light after 24-48 hours, oxidative-fermentative metabolism of glucose (LELLIOTT and STEAD, 1987); production of levan on NSA, presence of oxidase, potato soft rot, presence of arginine dihydrolase and hypersensitivity reaction (HR) on tobacco leaves - LOPAT tests (LELLIOTT *et al*., 1966) and gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and utilization of tartrate - GATTa tests (LATORRE and JONES, 1979).

PCR amplification and sequence analysis

Genomic DNA was extracted from 48 h old pure culture grown on NA medium according to the modified CTAB protocol described by AUSUBEL *et al*. (2003). Molecular identification was based on the detection of syringomycin synthesis (*syrB*) and syringomycin secretion (*syrD*) specific for *P. s.* pv. *syringae* simultaneously used by two sets of primers in m-PCR, B1/B2 and SyD1/SyD2 (SORESTEN *et al*., 1998; BULTREYS and GHEYSEN, 1999). Detection of coronatine production gene (*cfl*) was performed using Primer1/2 primer set specific for *P. s.* pv. *morsprunorum* race 1 (BERESWILL *et al*., 1994).

Multilocus sequence typing and analysis (MLST/MLSA) were performed by using partial sequences of *gapA* (glyceraldehyde-3-phosphate dehydrogenase A) gene (gapA-F: 5'-CGCCATYCGCAACCCG-3' / gapA-R: 5'- CCCAYTCGTTGTCGTACCA-3'), *gltA* (citrate synthase) gene (gltA-F: 5'- AGTTGATCATCGAGGGCGCWGCC-3' / gltA-R: 5'-TGATCGGTTTGATCTCGCACGG-3'), *gyrB* (DNA gyrase B) gene (GyrB-F: 5'-MGGCGGYAAGTTCGATGACAAYTC-3' / GyrB-R: 5'-TRATBKCAGTCARACCTTCRCGSGC-3') and *rpoD* (RNA polymerase sigma factor 70) gene (rpoD-F: 5'- AAGGCGARATCGAAATCGCCAAGCG-3' / rpoD-R: 5'-GGAACWKGCGCAGGAGTCGGCACG-3') (SARKAR and GUTTMAN, 2004; HWANG *et al*., 2005; YAN *et al*., 2008; FERRANTE and SCORTICHINI, 2010; BALAŽ *et al*., 2016; POPOVIĆ *et al*., 2019).

The PCR mix (25 µL) consisted of 12.5 µL of KAPA Taq ready mix (a doubly concentrated solution of Taq DNA polymerase in 0.05 U/µL of reaction buffer with a 0.4 mM concentration of each dNTP), 9.5 µL of ultrapure DNases/RNase-free water, 1 µL of each of the primers (10 µM) and 1 µL of the sample DNA. PCR amplification was performed according to the following protocol: initial denaturation step at 94°C for 3 min, then 30 cycles of denaturation at 94°C for 2 min, annealing at 54°C (*gapA*), 56°C (*gltA*), 62°C (*gyrB*) or 63°C (*rpoD*) for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. Obtained PCR products were checked for the expected-size band presence on 1% agarose gel stained with ethidium bromide. PCR products were purified with the QIAquick PCR purification kit (QIAGEN GmbH, Germany) and sequenced in Macrogen Sequencing Service (Amsterdam, the Netherlands). Considering the sequences homology among the each isolates group, sequenced nucleotides of three isolates representing each group were deposited into the GenBank database under accession numbers given in Table 1.

The obtained sequences were checked for quality, trimmed from the both ends to following sizes: 591 nt for *gapA*, 518 nt for *gltA*, 562 nt for *gyrB* and 481 nt for *rpoD* genes, and compared to the already deposited sequences in NCBI database by using the BLAST*n* tool. Sequences of different *P. syringae* pvs. strains were retrieved form the GenBank (Table 2) and aligned with the sequences of Serbian tested isolates using ClustalW segment of the BioEdit (ver. 7.0.5) program. The jModelTest v.2.0.2 (POSADA, 2008) following the Bayesian Information Criterion (BIC) was used to perform selection of the best evolutionary model of nucleotide substitution and the neighbour-joining method was selected to construct phylogenetic trees by Mega 7 (1,000 bootstrap replications) (TAMURA *et al.*, 2013). Phylogenetic trees were created for each gene separately and with concatenated sequences (2,152 nt). For their rooting strain *Xanthomonas campestris* pv. *campestris* ATCC 33913 retrieved from the GenBank was used as an outgroup strain.

To visualize phylogeographic insight among the tested Serbian isolates and strains from NCBI database, haplotype network was generated based on the concatenated sequences by using the TCS algorithm (CLEMENT *et al.*, 2002) implemented in PopART v 1.7 program (LEIGH and BRAYANT, 2015).

RESULTS AND DISCUSSION

During spring, in May 2018 and 2019 occurrence of bacterial canker symptoms was observed on a newly planted sweet cherry plantations on two localities, Žitorađa (2018) and Karavukovo (2019) and bacterial leaf spot in Žitorađa (2018). The most characteristic symptom where presence of bacterial canker occurred was leaf wilting, followed by dieback of young trees (Figure 1A-D). The infection started from the buds on stems, branches or from the place of heading cuts, followed with elongated, brown lesions with gum exudation (Figure 1A). When the bark was removed, a necrotic brown discoloured phloem and cambium were observed, and together with necrosis of xylem was manifested as long brown strips (Figure 1B). Girdling of the main stem dieback of whole tree occurred within weeks, and only the root remained healthy, as indicated by the outbreak of root suckers emerging from the base of the root. Bacterial leaf spot appeared in the form of brown-purple, round to angular spots, surrounded with yellow haloes (Figure 1C).

Table 2. Pseudomonas spp. *strains from NCBI database used for MLSA*

Strain	Country	Host	*gapA*	*gltA*	*gyrB*	*rpoD*
Pseudomonas syringae* pv. *syringae						
KBNS85	Serbia	Sweet cherry	KR051340	KR051312	KR051284	KR051368
KBNS93	Serbia	Sweet cherry	KR051348	KR051320	KR051292	KR051376
KBNS94	Serbia	Sweet cherry	KR051349	KR051321	KR051293	KR051377
H1	Hungary	Sour cherry	KR051350	KR051322	KR051294	KR051378
CFBP1608	France	*Rosa* sp.	HE604365	HE604380	HE604395	HE604410
NCPPB1652	South Africa	*Pisum sativum*	HE604359	HE604374	HE604389	HE604404
B301D	England	Diseased flower	CP005969	CP005969	CP005969	CP005969
HS191	Australia	*Panicum miliaceum*	CP006256	CP006256	CP006256	CP006256
IO110	Poland	Plant tissue	HG000157	HG000092	HG000222	HG000027
IO68	Poland	Plant tissue	HG000151	HG000086	HG000216	HG000021
IO210	Poland	Plant tissue	HG000163	HG000098	HG000228	HG000033
ITACyL	Spain	*Pisum sativum*	HE604362	HE604377	HE604392	HE604407
Pseudomonas cerasi						
PL963	Poland	Sweet cherry	LT963395	LT963395	LT963395	LT963395
***Pseudomonas syringae* pv. *morsprunorum* race 1**						
IO1061	Poland	Plant tissue	HG000209	HG000144	HG000274	LT963395
***Pseudomonas syringae* pv. *morsprunorum* race 2**						
IO732	Poland	Plant tissue	HG000197	HG000132	HG000262	HG000067
Pseudomonas syringae* pv. *avii						
CFBP 3846	France	Sweet cherry	LIIJ01000312	LIIJ01000301	JN190425	HG000079
Pseudomonas syringae* pv. *persicae						
NCPPB 2254	France	Peach	LAZV01000200	LAZV01000082	LAZV01000085	LAZV01000167

Due to development of numerous spots on leaves, centres of the necrotic spots drop out giving the leaf a shot hole appearance. The observed symptoms were similar to those caused by plant pathogenic bacterium *P. syringae* (KENNELLY *et al*., 2007; SPOTTS *et al*., 2010; KONAVKO *et al*., 2014; ILIČIĆ, 2016; BALAŽ *et al*., 2016; KALUZNA *et al*., 2016; ILIČIĆ *et al*., 2018).

Figure 1. Symptoms of bacterial canker and leaf spot on young sweet cherry trees. A: elongated, lesions with gum exudation, B: brown discolored phloem and cambium; C: bacterial leaf spot, the shot holes; D: dieback of young trees (photo T. Popović)

On NSA medium 2 days after incubation, large, convex, levan-type, mucoid, cream-whitish colonies were mainly formed. All tested isolates and reference strain KFB0103 caused symptoms in form of black, sunken lesions typical for *P. s*. pv. *syringae* on the inoculated immature sweet and sour cherry fruitlets. These results corresponded to other studies (LATORRE and JONES, 1979; BALAŽ *et al*., 2016). Fruitlets inoculated with SDW were symptomless.

Table 3. Biochemical characteristics of Serbian sweet cherry isolates and reference strains

Biochemical tests	Serbian *P. syringae* isolates													Reference strains			
	RE3	RE31	RE32	RE33	RE34	RE4	RE41	RE42	RE43	RE44	RE01	RE02	RE03	RE04	RE05	KFB 0103	KFB 0120	KFB 0121	KFB 0101
Levan production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Presence of oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pectinolytic activity on potato	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Presence of arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypersensitivity reaction on tobacco	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Tyrosine activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Utilization of L-tartrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+

+ positive reaction; - negative reaction

The results of biochemical tests for tested isolates and reference strains are shown in Table 3. All tested isolates were Gram-negative, catalase positive, produced fluorescent pigment on King's B medium and showed oxidative metabolism of glucose. Furthermore, levan production and tobacco hypersensitivity (HR) were positive, but oxidase, pectolytic activity and arginine dihydrolase were negative (LOPAT tests), placing them in fluorescent *Pseudomonas* Group Ia (LELLIOTT *et al*., 1966). According to GATTa test all isolates hydrolyzed gelatin and aesculin, and were tyrosinase and L-tartarate negative ($G^+A^+T^-Ta^-$), which corresponds to results obtained for *P. s.* pv. *syringae* reference strain KFB0103 and the previous statements of the other authors (LATORRE and JONES, 1979; VICENTE *et al*., 2004; ILIČIĆ, 2016). Pathogenicity test and phenotypic characteristics, LOPAT and GATTa tests clearly differentiate all 15 isolates to belong to *P. s.* pv. *syringae.*

Amplification of DNA product on positions 752 bp and 1040 bp in size, for genes *syrB* and *syrD,* respectively showed presence of syringomycin in all tested sweet cherry isolates and reference strain KFB0103 *P. s.* pv. *syringae*. Detection of coronatine production gene (*cfl*) on position 650 bp was positive only for reference strain KFB0120 *P. s.* pv. *morsprunorum* race 1 (*cfl* +). For other reference strains *morsprunorum* race 1 (KFB0121 *cfl*-) and *morsprunorum* race 2 (strain KFB0101) none of the mentioned genes was amplified.

In PCR performed for MLST/MLSA, all 15 isolates produced bands of the expected size of 634 bp for *gapA*, 556 for *gltA*, 610 bp for *gyrB* and 521 bp for *rpoD*. After products sequencing, NCBI BLAST*n* identified all 15 isolates as *P. s.* pv. *syringae*. The five isolates from Karavukovo (2019) showed 100% identity with *P. s.* pv. *syringae* strain H1 obtained from sour cherry in Hungary for *gapA, gltA* and *gyrB* genes, and 99.38% identity for *rpoD* gene. Ten isolates from Žitorađa (2018) showed 100% identity for *gapA* gene with *P. s.* pv. *syringae* strain H1 (sour cherry, Hungary), 99.81% for *gltA* gene with *P. s.* pv. *syringae* strains KBNS83, KBNS93 and KBNS94 isolated from sweet cherry in Serbia, 99.47% identity for *gyrB* gene with *P. s.* pv. *syringae* strain CFBP2118 isolated from sweet cherry in France, and 99.58% for *rpoD* gene with *P. s.* pv. *syringae* strain CFBP1608 isolated from *Rosa* sp. in France.

Estimation of the best substitution model in jModelTest for the *P. s.* pv. *syringae* phylogeny was done for each gene based on 27 *P. s.* pv. *syringae* analysed sequences (15 Serbian isolates obtained from Žitorađa and Karavukovo and 12 NCBI strains). Used substitution model was Felsenstein (F81) (FELSENSTEIN, 1981) suggested by BIC.

Phylogenetic analysis applied by neighbour-joining method and based on individual *gapA, gltA, gyrB* and *rpoD* gene sequences grouped isolates from Žitorađa in one tree cluster, separated from the isolates originated from Karavukovo, demonstrating the presence of two genetically diverse groups of isolates (Figure 2).

A neighbour-joining phylogenetic tree constructed with concatenated sequences of the tested Serbian sweet cherry isolates and those of *P. syringae* pvs. strains from NCBI from Table 2 shows five closely related phylogenetic groups (Figure 3). The first group was consisted of ten Serbian isolates originated from Žitorađa and two *P. s.* pv. *syringae* strains CFBP1608 (*Rosa* sp., France) and IO210 (plant tissue, Poland). Three *P. s.* pv. *syringae* strains retrieved from NCBI KBNS85, KBNS93 and KBNS94 isolated in 2012 from sweet cherry in Serbia in locality Gornji Tavankut (BALAŽ *et al*., 2016), formed the second group and were positioned closely between isolates from Žitorađa and Karavukovo. The third group was consisted of all five isolates from

Karavukovo together with *P. s.* pv. *syringae* strains ITACyL P275 (*Pisum sativum*, Spain), H1 (sour cherry, Hungary) and IO68 (plant tissue, Poland). The other *P. s.* pv. *syringae* strains from NCBI database i.e. NCPPB1652 (*Pisum sativum*, South Africa), B301D (diseased flower, England), HS191 (*Panicum miliaceum*, Australia), and IO110 (plant tissue, Poland) were placed in the remaining two groups (4th and 5th group). The rest of used strains, *P. cerasi* Pl963, *P. syringae* pv. *morsprunorum* race 1 IO1061, *P. syringae* pv. *morsprunorum* race 2 IO732, *P. syringae* pv. *avii* CFBP3846, *P. syringae* pv. *persicae* NCPPB2254 on phylogenetic tree were clearly separated from Serbian isolates and *P. s.* pv. *syringae* strains from NCBI (Figure 3).

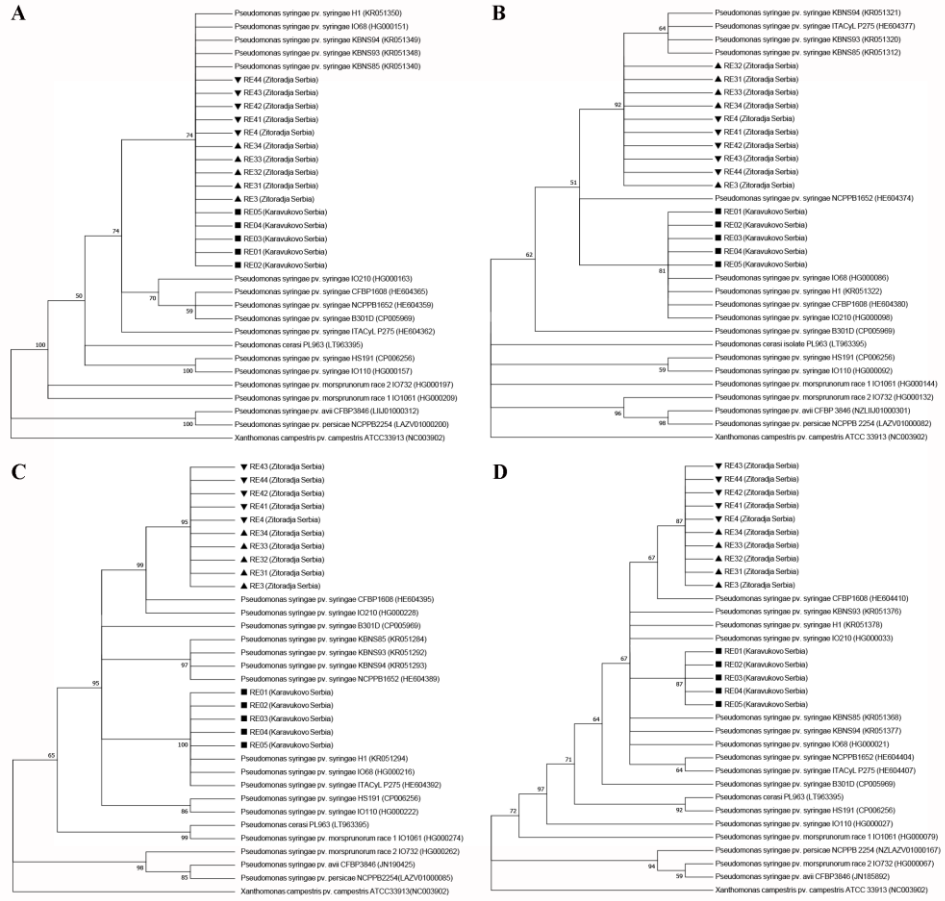

Figure 2. Neighbour-joining phylogenetic trees based on the individual sequences of *gapA* (A), *gltA* (B), *gyrB* (C) and *rpoD* (D) from Serbian tested sweet cherry isolates (marked with rectangle for locality Karavukovo and with upright (bacterial canker) and inverted (bacterial leaf spot) triangle for locality Žitorađa) and closest *Pseudomonas* sp. deposited in NCBI database. The trees were rooted with *X. c. pv. campestris* ATCC 33913 as an out-group strain.

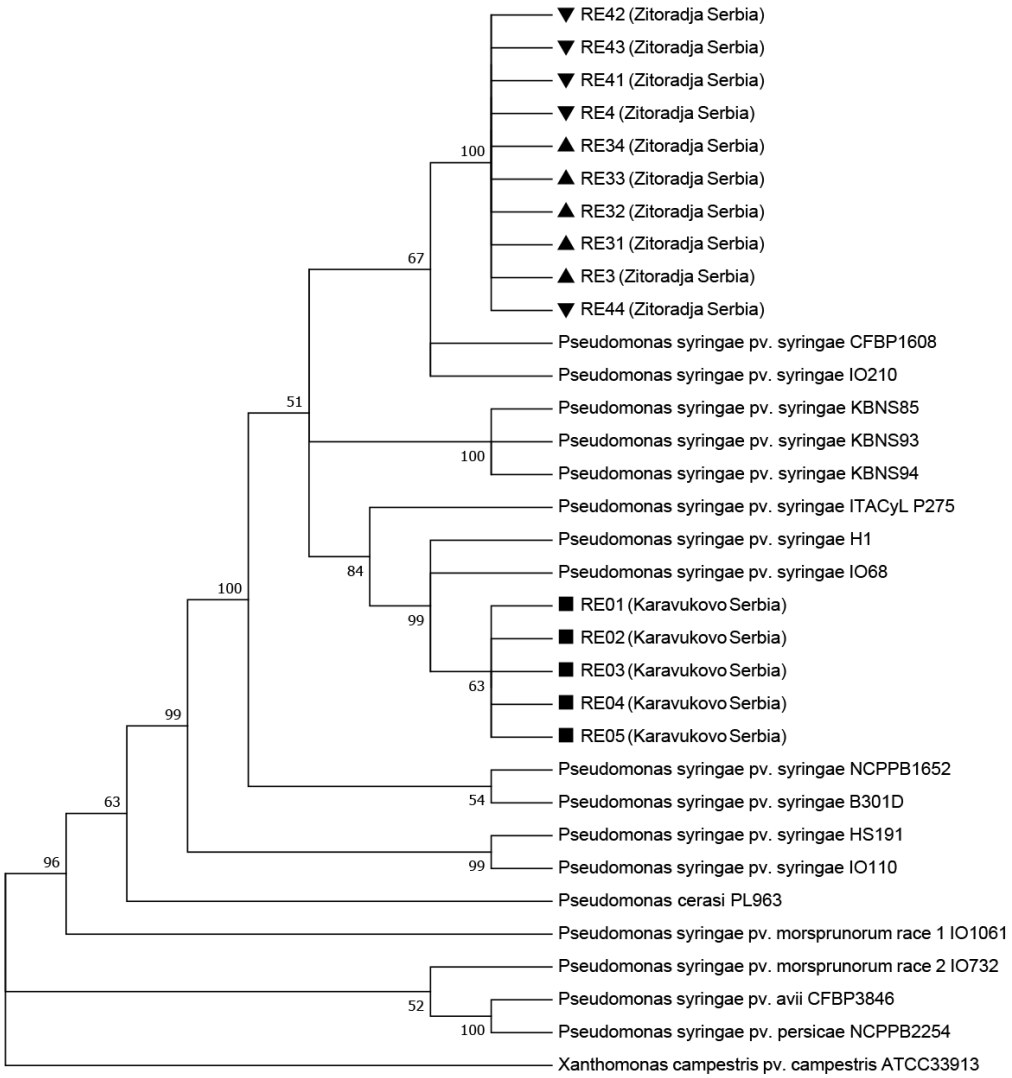

Figure 3. Neighbour-joining phylogenetic tree based on the concatenated sequences of *gapA*, *gltA*, *gyrB* and *rpoD* from Serbian tested sweet cherry isolates (marked with rectangle for locality Karavukovo and with upright (bacterial canker) and inverted (bacterial leaf spot) triangle for locality Žitorađa) and closest *Pseudomonas* sp. deposited in NCBI database. The tree was rooted with *X. c. pv. campestris* ATCC 33913 as an out-group strain.

Usage of MLST and MLSA to measure recombination rate and detect presence of genetic diversity within the group of *P. syringae* isolates was also described earlier (SARKAR and GUTTMAN, 2004; HWANG *et al*., 2005). Changes in the population structure of *P. syringae* on the genetic level are probably caused by high bacterial adaptability as well as major exchanges of sweet cherry planting material from the different geographic areas. MLSA, based on four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) have already been used to explore the diversity of *P. syringae* (HWANG *et al*., 2005; FERRANTE and SCORTICHINI, 2010; BALAŽ *et al*., 2016; POPOVIĆ *et al*., 2019).

Serbian sweet cherry isolates originated from Žitorađa were identified as the same multilocus haplotype of *P. s.* pv. *syringae* coded as REz. Isolates originated from Karavukovo were identified as same multilocus haplotype and coded as REk. The TCS network showed high genetic divergence between two Serbian haplotypes REz and REk (Figure 4). They are placed on ends of TCS network edge, while sweet cherry strains isolated in 2012 in Gornji Tavankut (Serbia) (BALAŽ *et al*., 2016) were centrally positioned in TCS network. These results indicate that geographic origin of isolates is not in correlation with their genetic similarity. It could be explained by the exchange of plant material among countries.

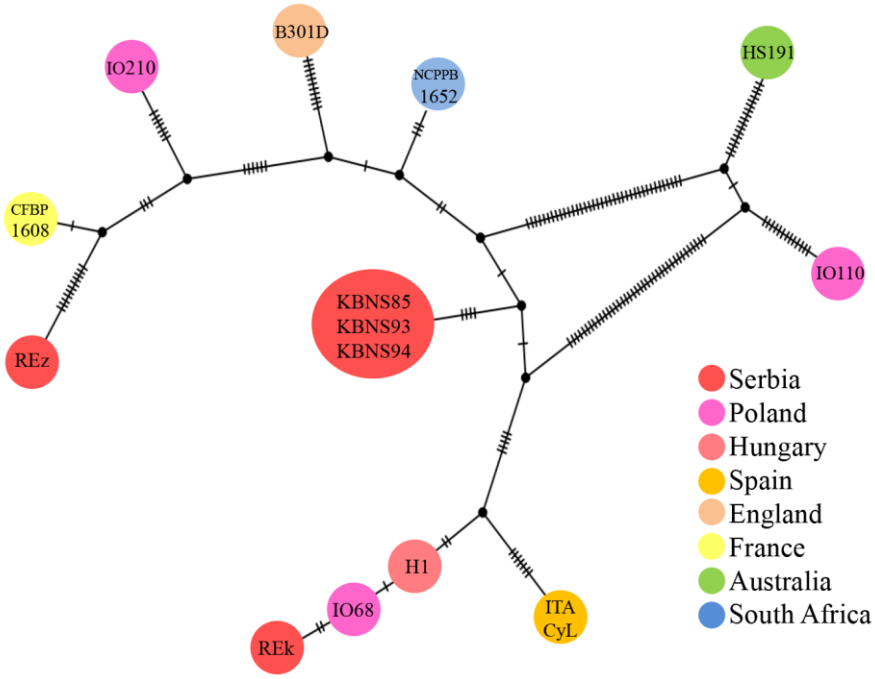

Figure 4. TCS network obtained for the *P. s.* pv. *syringae* haplotypes. Circle sizes are proportional to the number of strains included in a specific haplotype. Each black dash on the lines connecting the haplotypes marks one mutation. Black interconnecting dots are median vectors that represent missing or unsampled intermediate haplotypes different by a single mutation.

During the long time survey period it was noticed that bacterial canker caused by different *P. syringae* pvs. in Serbia frequently occurs on young sweet cherry trees where imported material was used for raising (ILIČIĆ, 2016). Appearance of bacterial canker is contributed to various factors such as increasing the area under sweet cherries, new cultivars and rootstocks, dense planting, heading cut, transport of trees and planting on permanent place, stress conditions (hail, drought, freezing) as well as epiphytic nature of the *P. syringae* pathogen (BALAŽ *et al*., 2016; ILIČIĆ *et al*., 2018, 2019).

Appearance of bacterial canker on cherries in the first year after raising plantations indicates latent infections of planting material as the main source of bacteria inoculum. Since the pathogen control is extremely difficult and losses are remarkable, it could be suggested to inspect the planting material from the import for the presence of *P. syringae* pvs. and to continuously survey sweet cherry health status in young plantations, starting immediately after planting until third year of vegetation.

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GENETIČKI DIVERZITET *Pseudomonas syringae* PV. *syringae* SA TREŠNJE U JUŽNOM I SEVERNOM REGIONU SRBIJE

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Izvod

Bakteriozno sušenje i pegavost lista prouzrokovano bakterijom *Pseudomonas* jedno je od najdestruktivnijih bolesti trešnje u svetu. Danas se u Srbiji proizvodnja trešnje značajno povećala, a nove plantaže sa sadnim materijalom iz uvoza, podižu se svake godine. Tokom proleća, u maju 2018. i 2019. godine, na novo podignutim plantažama trešnje na dva lokaliteta, Žitorađa (Južni region) i Karavukovo (Severni region-Vojvodina), primećena je pojava bakterioznog sušenja i lisne pegavosti. Kolonije tipične za *P. syringae* izolovane su na NSA iz svih prikupljenih uzoraka trešnje sa simptomima bakterioznog sušenja i lisne pegavosti. Za identifikaciju je odabrano ukupno 15 izolata. Rezultati LOPAT (+---+) testova potvrdili su da izolati pripadaju grupi fluorescentnih bakterija *Pseudomonas* grupa Ia, dok su prema $G^{+}A^{+}T^{-}Ta^{-}$ testovima izolati pokazali karakteristike *Pseudomonas syringae* pv. *syringae.* Patogenost je potvrđena na zelenim plodovima trešnje i višnje, na kojima su svi izolati prouzrokovali crne, ulegnute pege. Geni *syrB* i *syrD* uspešno su detektovani kod svih izolata. Sekvenciranjem *gapA, gltA, gyrB* i *rpoD* visokokonzervativnih gena korišćenjem NCBI BLAST svi izolati su identifikovani kao *P. s.* pv. *syringae.* Izolati su pokazali 99.47% do 100% (Žitorađa) i 99.38% do 100% (Karavukovo) identitet sa bakterijom *P. s.* pv. *syringae*. Filogenetska analiza grupisala je izolate iz Žitorađe u jedan klaster, odvojen od izolata poreklom iz Karavukova, što je pokazalo prisustvo dve genetski različite grupe izolata *P. s.* pv. *syringae,* dobijenih sa dva geografski različita lokaliteta u Srbiji. Filo-geografska analiza je grupisala izolate poreklom iz Žitorađe u *P. s.* pv. *syringae* multilokus haplotip označen kao REz i isolate poreklom iz Karavukova u multilokus haplotip označen kao REk. Obzirom sa se poslednjih nekoliko godina *P. syringae* uglavnom javlja u mladim plantažama trešnje, gde se uvozni material koristi za podizanje zasada, provera zdravstvenog stanja treba da bude uključena kao obavezna mera kada se sadni materijal koristi iz uvoza.

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