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Molecular characterization of *Pseudomonas syringae* pvs. from different host plants by repetitive sequence-based PCR and multiplex-PCR

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Abstract

Pseudomonas syringae pvs., isolated from different cultivars of sweet cherry grown in several locations in Serbia, were characterized and compared with strains collected earlier from sour and sweet cherry and oil pumpkin growing in this region, as well as with reference strains *P. syringae* pv. *morsprunorum* race 1 CFBP2119, *P. s*. pv. *lachrymans* 765R and *P. s*. pv. *syringae* H-1. By employing LOPAT and GATTa tests, isolates were identified as *P. syringae* pv. *syringae* and *P. s*. pv. *morsprunorum* race 1. Simultaneous detection of syringomycin synthesis (*syrB*) and syringomycin secretion (*syrD*) genes in multiplex-polymerase chain reaction (m-PCR) was used for *P. syringae* pv. *syringae* confirmation. All isolates detected as *P. syringae* pv. *morsprunorum* race 1 after biochemical characterization were positive for *cfl* gene amplification. Using a repetitive sequence-based PCR (rep-PCR) both *syringae* and *morsprunorum* race 1 pathovars were clustered separately, with 42% similarity. No significant differences between isolates in each pathovar were noted, although they were collected from different sweet cherry cultivars and varying localities. The most similar to the *P. syringae* pv. *syringae* isolates was strain T6 with 19% similarity, followed by strain Tk21 from oil pumpkin – 25%. The isolates of both pathovars were detected on the same location (Selenca, Serbia) and the same cultivar ('Merchant'), albeit in two different years.

Key words: oil pumpkin, sour cherry, sweet cherry, *cfl*, *syrB*, *syrD*.

Introduction

Pseudomonas syringae (van Hall) 1902 is polyphagous and widely spread species of phytopathogenic bacteria globally as well as in Serbia. Pathovars of *P. syringae* are very important causal agents of bacterial diseases in numerous woody and herbaceous plants. Based on the pathogenic characteristics and host range bacteria, *P. syringae* is divided into more than 60 pathovars, and on the basis of DNA homology, nine genomospecies can be distinguished (Gardan et al., 1999). In Serbia, *P. syringae* pv. *syringae* was determined as a pathogen for apricot, sweet and sour cherry, pear, apple, plum, peach, raspberry and many other herbaceous plants. The pathovar *P. syringae* pv. *morsprunorum* Wormald (1931) is also present on stone fruits (Gavrilović et al., 2012; Ivanović et al., 2009; 2012). Based on bacteriophage typing, two races of *P. s*. pv. *morsprunorum* have been described, namely race 1 which is pathogenic to cherry, plum and apricot, and race 2 that infects cherry only (Bultreys, Kaluzna, 2010). Host range of bacteria *P. syringae* pv. *syringae* in Serbia continues to expand on new plant species and recently Balaž et al. (2014) have determined oil pumpkin (*Cucurbita pepo* L.) as a new host. Oil pumpkin is a special form of ordinary pumpkin and has recently become an increasingly important source of high-quality oil rich seeds. Diseases common to pumpkin varieties are presently poorly investigated in Serbia. However, empirical evidence indicates that increased production has been accompanied by more frequent appearance of leaf spots at the beginning of the growing season, caused by *P. syringae* pv. *syringae.* Based on the classical bacteriological test LOPAT (production of levan, hypersensitivity to tobacco leaves, and presence of oxidase, potato soft rot and arginine dihydrolase) bacteria *P. syringae* belong to fluorescent *Pseudomonas* Group Ia (Lelliott et al., 1966). GATTa tests (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and utilization of tartrate) clearly differentiate *P. syringae* pv. *syringae* ($G^+A^+T^-Ta^-$), *P. syringae* pv. *morsprunorum* race 1 ($G^-A^-T^+Ta^+$) and *P. syringae* pv. *morsprunorum* race 2 ($G^+A^-T^-Ta^-$) (Latorre, Jones, 1979). The fact that *P. syringae* produce several well-characterized phytotoxic compounds specific to the

pathovar was explored as a viable method for their identification. The genes for syringomycin synthesis (*syrB*) and syringomycin secretion (*syrD*) are specific to the *P. syringae* pv. *syringae*, whereas coronatine production gene (*cfl*) is specific to the *P. syringae* pv. *morsprunorum* race 1 (Kaluzna et al., 2012). Because multiplex-polymerase chain reaction (m-PCR) allows the amplification of more than one target region in one PCR reaction mixture and can reduce the time and labour input compared with single PCR, this method has previously been employed for detection of a number of pathogens, including *Clavibacter michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato*, *P. savastanoi* pv. *savastanoi* and *Xanthomonas axonopodis* pv. *vesicatoria* (Özdemir, 2009). In our study, m-PCR was utilized to facilitate detection of genes for syringomycin synthesis and secretion (*syr*B and *syr*D) and to characterize isolates of *P. syringae* pvs. from sweet cherry. *P. syringae* pv. *syringae* are polyphagous bacteria. Moreover, due to the spreading on the new host, it is important to determine genetic heterogeneity between *P. syringae* pv. *syringae* strains originating from different and the same hosts. The genetic heterogeneity of *P. syringae* pv. *syringae* strains was examined by many authors (Vicente, Roberts, 2007; Abbasi et al., 2011; Dariush et al., 2012). Various molecular techniques have been used to characterize *P. syringae* pv. *syringae* strains, with the findings revealing high genetic heterogeneity between *P. syringae* pv. *syringae* strains originating from different and the same hosts (Natalini et al., 2006; Gilbert et al., 2009; Kaluzna et al., 2010; Ivanović et al., 2012). Repetitive element sequence-based PCR (rep-PCR) is based on three main sets of repetitive DNA elements: the repetitive extragenic palindromic (REP) elements (REP-PCR), the enterobacterial repetitive intergenic consensus (ERIC) sequences (ERIC-PCR) and BOX elements (BOX-PCR), and this method is successful in typing a variety of bacteria, as well as *P. syringae* (Vicente, Roberts, 2007; Marques et al., 2008; Kaluzna et al., 2010).

The aim of this study was to characterize and compare *P. syringae* pvs. strains originating from sweet cherry, sour cherry and oil pumpkin using rep-PCR. In order to facilitate detection of genes for syringomycin synthesis and secretion (*syr*B and *syr*D), respectively, the combination of two sets of primers in the same reaction was used in m-PCR.

Material and methods

The 155 isolates of *Pseudomonas syringae* employed in this study were obtained from diseased branches (cankers) and leaves (bacterial spots) of sweet cherry from several localities in Vojvodina during 2012, 2013 and 2014. In addition, eight isolates from oil pumpkin were collected near Bački Petrovac (experimental fields, Institute of Field and Vegetable Crops) during the 2008–2013 period and were subsequently identified as *P. syringae* pv. *syringae* (Balaž et al., 2014). In this study, only representative isolates were used (Table). We also included strains *P. syringae* intermediate forms V-85 (fruit) (Sabac) from sour cherry and one from sweet cherry V-109 (canker) (Subotica). In addition, French collection of plant pathogenic bacteria CFBP2119 (*P. syringae* pv. *morsprunorum*) and 765R (*P. syringae* pv. *lachrymans*) were used as reference strains. The strains *P. syringae* pv. *syringae* H-1 from sour cherry and T6 (were also used for comparison. LOPAT (levan production, oxidase response, potato tuber rot, presence of arginine dihydrolase, hypersensitive reaction in tobacco leaves) tests according to Lelliott et al. (1966) and GATTa (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and utilization of tartrate) according to Latorre and Jones (1979) were performed.

Bacterial strains were grown on King B medium, at 26°C for 24 h. The cells were boiled for 10 min, cooled on ice, and centrifuged at 13000 rpm for 3 min, after which the supernatant with total DNA extracted was kept at −20°C.

Two sets of primers were simultaneously used in m-PCR: B1 (CTTTCCGTGGTCTTGATGAGG) and B2 (TCGATTTTGCCGTGATGAGTC) specific to *syr*B gene, and SyD1 (CAGCGGCGTTGCGTCCATTGC) and SyD2 (TGCCGCCGACGATGTAGACCAGC), specific to *syr*D gene. Those primer sets were previously used in a separate polymerase chain reaction (PCR) during *P. s.* pv. *syringae* testing (Natalini et al., 2006; Gašić et al., 2012; Ivanović et al., 2012). PCR amplification was carried out in a 30 µl reaction volume using GreenTaq Dream Master Mix (Thermo Scientific, Lithuania) with 1.2 µl of template DNA and 50 pmol of each primer (B1/B2 and SyD1/SyD2) (Metabion, International AG, Germany). The program was comprised of 5 min of initial denaturation at 94°C, followed by 35 cycles of 1.25 min at 94°C, 1.25 min at 61°C, 2 min at 72°C and a 7 min of final extension at 72°C.

Gene *cfl* (coronatine production) is specific to the *P. s*. pv. *morsprunorum* race 1 and produces an amplicon of 650 bp using primer set Primer1/2 (Metabion International AG, Germany). Amplification conditions adopted in this work followed the recommendations of Gašić et al. (2012). PCR amplification was carried out in a 30 µl reaction volume using GreenTaq Dream Master Mix (Thermo Scientific, Lithuania) with 1.2 µl of template DNA and 50 pmol of primer1/primer2. The program was comprised of 2 min initial denaturation at 93°C, followed by 37 cycles of 2 min at 93°C, 1 min at 67°C, and 2 min at 72°C, with a 10 min of final extension at 72°C.

rep-PCR analysis was performed using two primer sets for REP (REP IR-I/REP 2-I) and ERIC (ERIC1R/ERIC2) and two primers for BOX-PCR (BOX A1R and $(GTG)_5$), as recommended by Marques et al. (2008) and Gilbert et al. (2009). Amplification was conducted in 25 µl of reaction mixture containing GreenTaq Dream Master Mix (Thermo Scientific, Lithuania), 50 pmol of each primer, 5% DMSO (dimethylsulfoxide) and 1.3 µl

Table. Origin and characterization of *Pseudomonas syringae* (*P. s.*) isolates and list of reference strains

Strain / Isolate	Host	Cultivar	Plant organ	Locality	Year of isolation	*P. s.* pathovar[1]	m-PCR (*syr*B / *syr*D)	*cfl*	rep-PCR pattern[2]
V-109	sweet cherry	nd	branch	Subotica	1988	intermediate form	+/+	−	a
V-85	sour cherry	nd	fruit	Sabac	1988	intermediate form	+/+	−	b
KBNS71	sweet cherry	Burlat	branch	Selenca	2012	*morsprunorum* race 1	−/−	+	c
KBNS93	sweet cherry	Summit	branch	Gornji Tavankut	2012	*syringae*	+/+	+	d
S1	sweet cherry	Burlat	branch	Selenca	2013	*morsprunorum* race 1	−/−	+	c
S15	sweet cherry	Merchant	branch	Selenca	2013	*morsprunorum* race 1	−/−	+	c
S34	sweet cherry	Vanda	branch	Selenca	2014	*morsprunorum* race 1	−/−	+	c
S41	sweet cherry	Vanda	leaf	Selenca	2014	*morsprunorum* race 1	−/−	+	c
M4	sweet cherry	Kordia	branch	Mikicevo	2014	*morsprunorum* race 1	−/−	+	c
S30	sweet cherry	Merchant	branch	Selenca	2014	*syringae*	+/+	−	d
T2	sweet cherry	Summit	branch	Gornji Tavankut	2013	*syringae*	+/+	−	d
K1	sweet cherry	Valeri Chkalov	steam	Kanjiza	2013	*syringae*	+/+	−	d
LJ1	sweet cherry	Germerzdorfer	branch	Ljutovo	2014	*syringae*	+/+	−	d
M1	sweet cherry	Regina	branch	Mikicevo	2014	*syringae*	+/+	−	d
Tk21	oil pumpkin	Olinka	leaf	Backi Petrovac	2008	*syringae*	+/+	−	e
Reference strains									
CFBP2119	Collection Française des Bactéries Phytopathogènes					*P. s.* pv. *morsprunorum* race 1	−/−	+	f
765R	cucumber	nd	leaf	Ohio	–	*P. s.* pv. *lachrymans*	−/−	−	g
H-1	sour cherry	nd	–	–	–	*P. s.* pv. *syringae*	+/+	−	h
T6	sweet cherry	nd	branch	–	–	*P. s.* pv. *syringae*	+/+	−	i

Note. KBNS – Kolekcija Bakterija Novi Sad; [1] – pathovar identification on the basis of LOPAT and GATTa tests; [2] – the same rep-PCR patterns are marked with the same letter based on cumulative analysis of REP, ERIC, BOXA 1R and $(GTG)_5$ patterns; nd – no data.

of template DNA. The amplification conditions for REP-PCR consisted of an initial denaturation at 95 for 4 min, followed by 30 cycles (denaturation at 94°C for 55 s, primer annealing at 42°C for 65 s and primer extension at 65°C for 7 min) and a final extension at 65°C for 12 min. ERIC and BOX were conducted using the program comprising 3 min initial denaturation at 95°C, followed by 32 cycles of 1 min denaturation at 94°C, 1 min at 52°C (ERIC) or 53°C (BOX) for primer annealing and 8 min at 65°C for primer extension, followed by a 16 min of final extension at 65°C.

All amplifications (rep-PCR and m-PCR) were performed in Eppendorf MasterCycler Personal (Germany) and were repeated at least twice for each bacterial strain. Amplified DNA products (8 µl) were separated by electrophoresis in 1.5% (w/v) agarose gel in 0.5X Tris-borate-EDTA buffer at 5 V cm^{-1} for 1.5 h (m-PCR) or 2.5 h (rep-PCR), after which they were stained with ethidium bromide (0.5 µg ml^{-1}) and photographed under UV transilluminator (Amercham Pharmacia Biotech, United Kingdom). The amplicon size was determined by comparison with GeneRuler DNA Ladder Mix SM0331 and SM1551 (Thermo Scientific, Lithuania) as the DNA molecular weight markers.

To compare bacterial strains on the basis of rep-PCR fingerprint, reproducible bands ranging in size from 250 bp to 3 kb were scored and converted into binary data. Cluster analysis was performed via the software *STATISTICA 12* using cumulative data from all amplifications (REP, ERIC, BOX A1R and $(GTG)_5$).

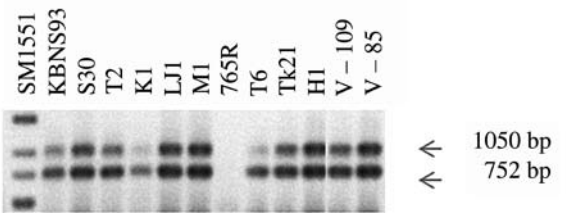
Results and discussion

In this work, isolates of *P. syringae* from sweet cherry, along with several isolates from oil pumpkin and sour cherry, were characterized on the basis of phenotypic and genotypic methods. Both method types were very useful for pathogen identification and characterization.

All investigated isolates from sweet cherry and Tk21 strain from oil pumpkin were aerobic, gram-negative, non-spore-forming rods. All isolates produced fluorescent pigment and catalase, as well as induced a hypersensitive reaction in tobacco leaves, while not causing soft rot of potato tubers. In addition, all were positive for levan and negative for oxidase and arginine dihydrolase. According to the LOPAT tests, these isolates were classified in the Ia subgroup of Pseudomonads. GATTa tests for pathovar differentiation of *P. syringae* (pv. *syringae* and pv. *morsprunorum*) revealed two

distinct isolate groups: group I $G^+A^+T^-Ta^-$ (KBNS93, S30, T2, K1, Lj1, M1) belonging to pathovar *syringae* and group II $G^-A^-T^+Ta^+$ (KBNS71, S1, S15, S34, S41, M4) that belong to pathovar *morsprunorum* race 1.

Genes *syrB* (syringomycin synthesis) and *syrD* (syringomycin secretion) specific to *P. s*. pv. *syringae* were simultaneously detected in isolates KBNS93, S30, T2, K1, Lj1, M1 and strain Tk21, amplifying DNA fragments of 752 and 1040 bp, respectively (Fig. 1). Based on the GATTa test results, these isolates and strain Tk21 were classified in *P. s*. pv. *syringae*. The *syrB* and *syrD* genes were also detected in the strains of intermediate forms V-109 and V-85, as well as in strain T6. Absence of *syrB* and *syrD* genes was noted in isolates KBNS71, S1, S15, S34, S41 and M4, all of which were classified in the *morsprunorum* race 1 on the basis of the GATTa test results. The amplification was positive for the

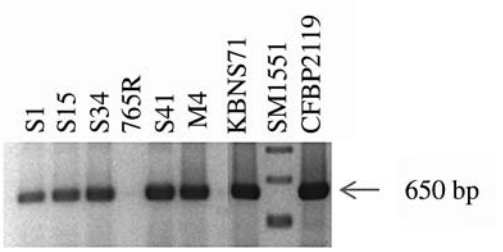


P. syringae pv. *syringae* isolates: KBNS93, S30, T2, K1, Lj1, M1 (all from sweet cherry), V-85 (sour cherry) and Tk21 (oil pumpkin); reference strains: 765R – pv. *lachrymans*; H-1 and T6 – pv. *syringae*; marker: SM1551 – GeneRuler DNA Ladder Mix (Thermo Scientific, Lithuania)

Figure 1. Detection of *syrB* and *syrD* genes in *Pseudomonas syringae* isolates originated from sweet cherry, sour cherry and oil pumpkin

reference strain H-1 and T6, which were used as positive control. On the other hand, it was negative for reference strains *P. syringae* pv. *morsprunorum* CFBP2119 and pv. *lachrymans* 765R, used as negative control. Available evidence confirms that m-PCR is a rapid identification procedure and is a very useful method when there is a need to avoid the physiological, biochemical, and pathological tests used to characterize the strains of phytopathogenic bacteria (Özdemir, 2009). A method by which simultaneous detection of both genes can be achieved successfully was used by Rico et al. (2006), and was applied in the identification of *P. s*. pv. *syringae* isolates originating from beans. The authors indicated that this simultaneous detection method, including a large number of genes, is precise and rapid, and this assertion was confirmed in our investigation. Separate application of primer genes for syringomycin synthesis and secretion was reported by several authors (Kaluzna et al., 2010; Bultreys, Kaluzna, 2010; Gašić et al., 2012; Ivanović et al., 2012). According to Bultreys and Kaluzna (2010), some *P. s*. pv. *syringae* isolates do not possess both *syrB* and *syrD*; however, this finding was not supported by the present study results.

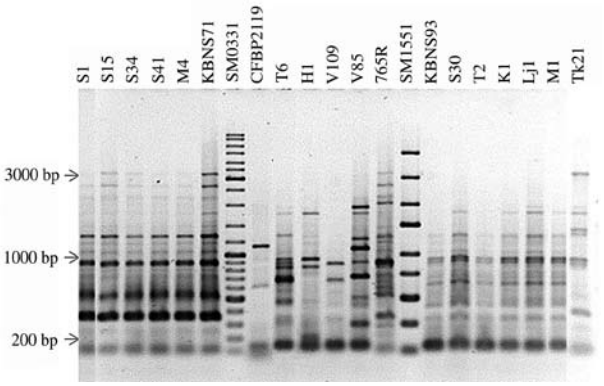
The expected fragment of 650 bp corresponding to the coronatine synthesis gene, specific to the *P. s*. pv. *morsprunorum* race 1, was detected in six isolates (KBNS71, S1, S15, S34, S41 and M4) and the reference strain CFBP2119 (Fig. 2). This finding is in agreement with the GATTa test results, while also confirming the absence of *syrB* and *syrD* genes in the aforementioned isolates. PCR detection of *cfl* gene in pv. *morsprunourm* race 1 was reported by Gilbert et al. (2009), Bultreys and Kaluzna (2010) and Kaluzna et al. (2010). According to these authors, while most of the pv. *morsprunorum* race 1 isolates possess the *cfl* gene, not all isolates of this pathovar have to possess this gene for coronatine synthesis. In this study, all investigated isolates identified as pv. *morsprunorum* race 1 by biochemical-physiological methods possessed the *cfl* gene, thus confirming that they belong to pv. *morsprunorum* race 1.



Reference strains: 765R – *P. syringae* pv. *lachrymans*; CFBP2119 – pv. *morsprunorum*; marker: SM1551 – GeneRuler DNA Ladder Mix (Thermo Scientific, Lithuania)

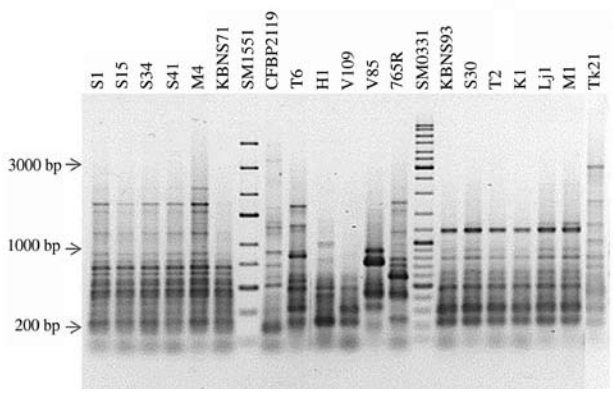
Figure 2. Detection of *cfl* gene in *Pseudomonas syringae* isolates originated from sweet cherry

To assess the extent of similarity between isolates from sweet cherry, strains from sour cherry and those of oil pumpkin, and the reference strains, rep-PCR were used. The obtained patterns are shown in Figures 3–6.



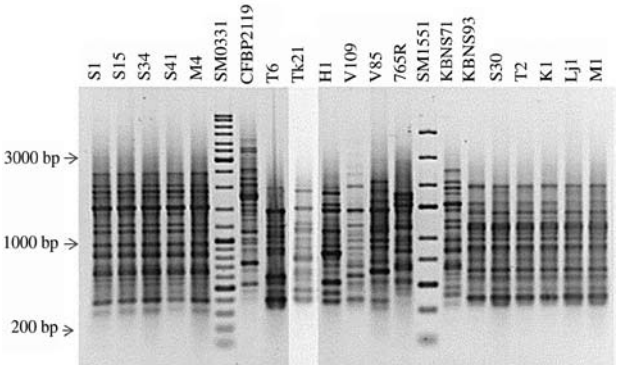
P. syringae pv. *syringae* isolates: KBNS93, S30, T2, K1, Lj1, M1 (all from sweet cherry), V-85 (sour cherry) and Tk21 (oil pumpkin); pv. *morsprunorum* race 1: S1, S15, S34, S41, M4, KBNS71 (all from sweet cherry); reference strains: H-1 and T6 – pv. *syringae*; pv. *morsprunorum* race 1: CFBP2119; pv. *lachrymans*: 765R; marker SM0331 – GeneRuler DNA Ladder Mix and Marker SM1551 GeneRuler Express DNA Ladder (Thermo Scientific, Lithuania)

Figure 3. REP-PCR of *Pseudomonas syringae* isolates originated from different hosts



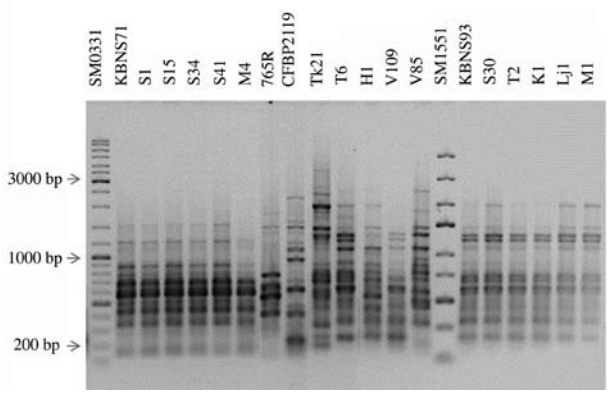
Explanation under Figure 3

Figure 4. ERIC-PCR of *Pseudomonas syringae* isolates originated from different hosts



Explanation under Figure 3

Figure 5. BOX-PCR: BOX 1R of *Pseudomonas syringae* isolates originated from different hosts

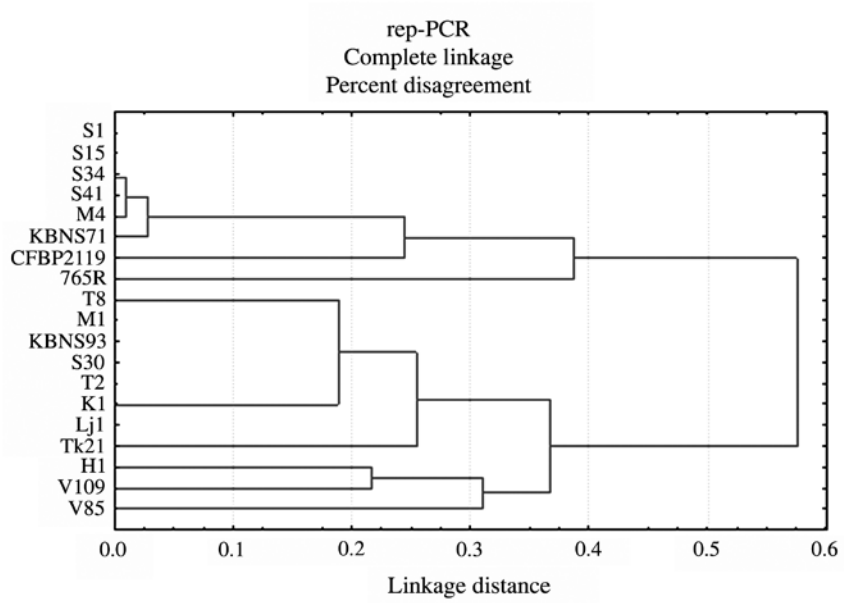


Explanation under Figure 3

Figure 6. BOX-PCR $(GTG)_5$ of *Pseudomonas syringae* isolates originated from different hosts

The bands from 200 to 3000 bp, representing REP, ERIC, BOXA 1R and $(GTG)_5$ amplifications, were included in the analysis and 106 bands were scored.

Cumulative cluster analysis showed nine different patterns at a similarity threshold of 90% (Fig. 7). Cumulative rep-PCR diagram consisted of two clusters that were 58% different. The first cluster consisted of six isolates and five strains and had two subclusters that were 37% distinct from one another. The first branch in the first subcluster contained only strain V-85, whereas the second branch included strains V-109 and H-1. Strain V-85 was 31% different from the other strains and isolates in this cluster. The separate branch in the other subcluster formed strain Tk21, with 25% differences compared to the other branch that included isolates KBNS93, LJ1,



P. syringae pv. *syringae* isolates from: a) sweet cherry – KBNS93, S30, T2, K1, Lj1 and M1; b) sour cherry – V-85; c) oil pumpkin – Tk21; pv. *morsprunorum* race 1 from sweet cherry: S1, S15, S34, S41, M4 and KBNS71; reference strains – H-1 and T6 – pv. *syringae*; pv. *morsprunorum* race 1 – CFBP2119, pv. *lachrymans* – 765R

Figure 7. Cumulative dendrogram of rep-PCR constructed on the basis of REP, ERIC, BOXA 1R and $(GTG)_5$ of *Pseudomonas syringae* originated from different hosts

K1, T2, S30, M1 and strain T6. Between strain T6 and other isolates, which were identical, a difference of 19% was identified. All of the above strains and isolates of this cluster belong to *P. s*. pv. *syringae*. The second cluster consisted of two subclusters. The first contained strain 765R (pv. *lachrymans*) only, which was 39% distinct from the other subcluster, which had two branches. One branch contained strain CFBP2119 only with a difference of 24% from the other branch, consisting of very similar or identical (97–100%) isolates, namely KBNS71, M4, S41, S34, S15 and S1. All isolates belong to pv. *morsprunorum* race 1, as does the strain CFBP2119. The isolates KBNS93 and KBNS71 collected in 2012 were selected and deposited in KBNS (Kolekcija Bakterija Novi Sad) collection as representative strains for pv. *syringae* and pv. *morsprunorum* from sweet cherry, respectively. In this work, rep-PCR clearly indicated the differences between pathovars *syringae* and *morsprunorum* race 1. Thus, this method is useful in the identification of these pathogens globally, and in our country, as was previously reported by a number of authors (Vicente, Roberts, 2007; Gilbert et al., 2009; Ivanović et al., 2009; 2012; Bultreys, Kaluzna, 2010; Kaluzna et al., 2010; Gašić et al., 2012; Gavrilović et al., 2012; 2013).

The cumulative rep-PCR dendrogram revealed 58% differences between isolate groups I and II. The tested isolates from sweet cherry of different origins and cultivars did not exhibit significant differences with respect to the same pathovar. Among the members of *P. s*. pv. *syringae*, six representative isolates and the strain T6, all from sweet cherry, showed the highest similarity (81%), while strain Tk21 from oil pumpkin was 75% similar. The reference strains H-1 and V-85 from sour cherries and V-109 from sweet cherry formed a separate subcluster, characterized by a lower similarity. Based on rep-PCR in pv. *syringae*, we identified the differences between representative isolates and strains from other location (Subotica) and times of isolation (1988) from the same host (sweet cherry), as well as differences in relation to other hosts (sour cherry and oil pumpkin). The pv. *syringae* was detected on sweet cherry from all tested localities, namely Selenca, Gornji Tavankut, Kanjiza, Ljutovo, Mikicevo, including Subotica, on sour cherry from Sabac and also on oil pumpkin from Backi Petrovac. Five cultivars were susceptible to this pathovar – 'Summit', 'Merchant', 'Regina', 'Valeri Chkalov' and 'Germerzdorfer'. Differences of less than 5% were observed in the group comprising the isolate pv. *morsprunorum* race 1 from sweet cherry and about 24% from the reference strain CFBP2119 of the same pathovar, which indicates low diversity in the populations of this pathogen. This pathovar was isolated from sweet cherry cultivars 'Burlat', 'Kordia', 'Vanda' and 'Merchant' grown on Selenca and Mikicevo. Both pathovars were detected on cv. 'Merchant' from Selenca in two successive years. The tested isolates exhibited slight heterogeneity of the population based on rep-PCR in the case of both pathovars, which is in agreement with the results reported by other authors. According to Vicente and Roberts (2007), using rep-PCR between isolates pv. *morsprunorum* race 1 and 2, a nearly homogeneous population was determined. On the other hand, greater genetic variability between pv. *syringae* isolates and sweet and wild cherries was noted. Gilbert et al. (2009) reported a similar finding following the identification of isolates pv. *syringae*, *morsprunorum* races 1 and 2 and pv. *avii*. Natalini et al. (2006) investigated isolates of pv. *syringae* strains originating from pear using REP-PCR and BOX noting presence of genetic diversity. However, some authors point out that the application of PCR using only one or two sets of primers is insufficient to reveal differences between pathogen isolates and races. On the other hand, Menard et al. (2003) posited that using ERIC and BOX-PCR helped differentiate pv. *persicae* from wild cherry isolates, which was not possible using REP-PCR. Strain 765R pv. *lacrymans*, used in this study as a reference strain, showed greater similarity to the isolates and strains CFBP2119 belonging to pv. *morsprunorum* race 1, grouping them in a separate cluster. Nonetheless, it was still separated into a distinct subcluster, which is consistent with the results reported by Menard et al. (2003). Based on the BOX-PCR, Ivanović et al. (2009) indicated that isolates of *P. syringae* pvs. in Serbia showed great variability depending on the host from which they were isolated. Gavrilović et al. (2012), following identification of bacteria *P. syringae* pvs. originating from sweet cherries, concluded that the use of REP-PCR detection methods provides reliable detection of pv. *morsprunorum* race 1 isolates. However, the authors also noted that genetic variability of pv. *syringae* isolates is greater than in the case of pv. *morsprunorum* isolates obtained from various locations in the Serbia, and thus requires usage of additional methods.

Conclusions

1. On the basis of LOPAT tests, strains were classified in the Ia subgroup of *Pseudomonas*. GATTa tests for the differentiation pathovars of *P. syringae* revealed two groups of isolates represented by KBNS93 for pathovar *syringae* and KBNS71 for pathovar *morsprunorum* race 1.

2. Detection of *syrB* and *syrD* in m-PCR confirmed *P. sringae* pv. *syringae* identification in seven representative isolates including Tk21, as well as in V-109 and V-85 isolates and strain T6. Negative results for amplification of *syrB* and *syrD* genes and positive for *cfl* gene confirmed identification of pv. *morsprunorum* race 1 for six representative isolates and reference strains pv. *morsprunorum* CFBP2119.

3. Using a rep-PCR (BOX, REP and ERIC) both *syringae* and *morsprunorum* race 1 pathovars clustered separately. However, isolates of both pathovars showed low population heterogeneity. *P. sringae* pv. *syringae* was detected on five sweet cherry cultivars from all tested

localities, while pv. *morsprunorum* race 1 was found on four cultivars from two localities. Both pathovars were detected on sweet cherry cv. 'Merchant' from Selenca, Serbia, in two different years.

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Įvairių augalų-šeimininkų *Pseudomonas syringae* pvs. molekulinis apibūdinimas taikant pasikartojančių sekų ir jungtinę PGR

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Santrauka

Pseudomonas syringae patotipai (pt), izoliuoti iš įvairių veislių trešnių, auginamų keliose vietovėse Serbijoje, buvo apibūdinti ir palyginti su anksčiau surinktomis iš trešnių, vyšnių ir aliejinių moliūgų, auginamų tame regione, padermėmis, taip pat su standartinėmis *P. syringae* pt. *morsprunorum*, rasė 1 CFBP2119, *P. syringae* pt. *lachrymans* 765R ir *P.* syringae pt. *syringae* H-1 padermėmis. Taikant LOPAT ir GATTa testus, izoliatai buvo identifikuoti kaip *P. syringae* pt. *syringae* ir *P. syringae* pt. *morsprunorum* rasė 1. Siekiant patvirtinti *P. syringae* pt. *syringae* taikyta jungtinė polimerazės grandininė reakcija (m-PGR), sinchroniškai nustatyti siringomicino sintezės (*syrB*) ir siringomicino sekrecijos (*syrD*) genai. Visi izoliatai, nustatyti kaip *P. syringae* pv. *morsprunorum* rasė 1, po biocheminio apibūdinimo amplifikavo geną *cfl*. Taikant kartotinėmis sekomis paremtą PGR (rep-PGR), ir *syringae*, ir *morsprunorum* rasės 1 patotipai grupavosi atskirai, tarp jų buvo nustatytas 42 % panašumas. Kiekviename patotipe tarp izoliatų esminių skirtumų nebuvo nustatyta, nors jie buvo surinkti iš įvairių veislių trešnių, augančių skirtingose vietovėse. Į *P. syringae* pt. *syringae* izoliatus panašiausia buvo padermė T6 – panašumas 19 % , po to padermė Tk21 iš aliejinių moliūgų – panašumas 25 %. Abiejų izoliatų patotipai nustatyti toje pačioje vietovėje (Selenca, Serbija) ir toje pačioje veislėje (‘Merchant’) skirtingais metais.

Reikšminiai žodžiai: aliejinis moliūgas, *cfl*, *syrB*, *syrD*, trešnė, vyšnia.

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