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Original scientific paper

Characterization and population diversity of *Erwinia amylovora* strains originating from pome fruits in Serbia

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SUMMARY

The diversity of 30 *Erwinia amylovora* strains, isolated from quince, pear and apple trees on 14 localities in Serbia, was studied using bacteriological and molecular methods. In pathogenicity tests, all strains caused necrosis and oozing of bacterial exudate on inoculated immature pear, cherry and plum fruits, and induced hypersensitive reaction in tobacco leaves. The studied strains were Gram and oxidase negative, non-fluorescent, levan and catalase positive and facultatively anaerobic. The strains did not reduce nitrates, but utilized citrate and produced acid from sorbitol, hydrolyzed gelatine, produced reducing substances from sucrose and grew in the presence of 5% NaCl, but not at 36°C. Identity of the strains was confirmed by conventional and nested PCR methods. Rep-PCR with REP, ERIC and BOX primers resulted in amplification of several DNA fragments respectively, but showed no variation within the strains. However, different genetic profiles were obtained with RAPD-PCR by using six primers which enabled differentiation of the strains into four groups. Genetic differences between the studied strains did not correlate with the host plants, geographical origin or year of isolation.

Keywords: Fire blight; Heterogeneity; Pathogenicity; RAPD-PCR; Rep-PCR; Pome fruits

INTRODUCTION

Fire blight, caused by *Erwinia amylovora* (Burrill) Winslow et al., is among the most destructive bacterial diseases of pome fruits and some ornamental plants (Van der Zwet & Keil, 1979). The disease was observed in Serbia for the first time in 1989, and the pathogen was officially confirmed in pear and quince trees in the vicinity of Šabac in 1990 (Arsenijević et al., 1991). Although eight new hosts have been detected in Serbia since then, the bacterium causes greatest economic damage to pear, apple and quince production (Arsenijević & Gavrilović, 2007). Previous studies had indicated that *E. amylovora* strains from Europe constitute a homogeneous group with low genetic variability

(Pulawska & Sobiczewski, 2012; Rezzonico et al., 2016). However, due to the development of genome analysis techniques, some differences among strains have been discovered in recent years (Pulawska & Sobiczewski, 2012). The PFGE has been used for differentiation of *E. amylovora* strains, as well as for monitoring of introductions and spread of the pathogen (Jock et al., 2002; Donat et al., 2007). This method revealed at least two possible directions of *E. amylovora* introduction into Serbia (Ivanović et al., 2012).

Rep-PCR and RAPD-PCR techniques are suitable for determination of genetic diversity and differentiation between strains of various bacterial species. Rep-PCR was one of the first techniques used for assessment of genetic diversity of *E. amylovora* strains, which enabled differentiation of strains originating from pome fruits and those isolated from raspberry and blackberry (McManus & Jones, 1995). This technique was applied both in diagnostics and in epidemiological studies (Versalovic et al., 1991; Louws et al., 1999). The RAPD-PCR was initially applied by Momol et al. (1997), enabling separation of two host-range groups (*Pomoideae* and *Rubus*) and one geographical region group (Hokkaido) among *E. amylovora* strains. Although this technique was useful in differentiation of *E. amylovora* strains from Ireland (Brennan et al., 2002), it revealed high homogeneity among *E. amylovora* strains from Israel (Manulis-Sasson et al., 1998), Australia (Taylor & Hale, 1998), Austria and Hungary (Keck et al., 2002) and Poland (Pulawska et al., 2006).

So far, the diversity of *E. amylovora* population from Serbia has been studied by PFGE and automated techniques, such as Biolog^{TM} and fatty acid analysis (Ivanović et al., 2012). The objective of this research was to study the population with other molecular methods, such as REP, ERIC and BOX-PCR and by using six primers in RAPD-PCR. These techniques could further discover possible genetic differences between the strains recently isolated in Serbia, as well as their correlations with hosts, year of isolation or localities from which they were isolated.

MATERIAL AND METHODS

Bacterial strains

Thirty strains of *E. amylovora* were isolated from apple, pear and quince from 14 different locations in Serbia, during 2007-2014 (Table 1). In all tests, 24h-old cultures were used, grown on nutrient agar (NA) (Torlak, Belgrade) or King's medium B (KB) at 27°C. During the study, bacterial cultures were stored in sterile distilled water in micro tubes at 4°C, or in LB medium with 20% glycerol in cryotubes at -20°C (Schaad, 2001).

Bioassays and pathogenicity test

Strain pathogenicity was tested on immature pear (cv. 'Santa Maria'), cherry (cv. 'Summit') and plum (cv. 'Stanley') fruits. The fruits were washed in tap water and surface disinfected with 70% ethanol. Inoculation was carried out by pricking the fruits with a disposable pipette tip, and leaving 10 µl of the bacterial suspension at the point of inoculation. Inoculum was prepared by suspending bacteria in sterile distilled water up to concentration of 10^8 CFU/ml. Inoculated fruits were placed into plastic boxes with wet filter paper to provide increased humidity, and incubated at room temperature. Development of symptoms on fruits was evaluated three and five days after inoculation. The same bacterial suspension was used for a tobacco (cv. 'Banat') hypersensitive reaction (HR) test. The suspension was infiltrated into the intercostal leaf tissue with a syringe and hypodermic needle. Necrosis of the infiltrated leaf area within 24 h was considered a positive reaction. In both tests, reference *E. amylovora* strains NCPPB 595 and CFBP 1430 were used as positive controls, and sterile distilled water as negative control.

Biochemical and physiological characteristics

In order to identify the strains, the following biochemical and physiological characteristics were studied: Gram reaction, levan production, fluorescence on KB, oxidase and catalase activity, oxidative/fermentative metabolism (O/F test), acid production from glucose, metabolism of sorbitol, nitrate reduction, production of reducing substances from sucrose, gelatine liquefaction, citrate utilization from Simmons' medium, growth at 36°C and tolerance to 5% NaCl (Fahy & Hayward, 1983; Lelliott & Stead, 1987; Klement et al., 1990; Arsenijević, 1997; Schaad, 2001). Colony morphology was observed on nutrient agar amended with 5% sucrose (NSA) and KB medium after 24, 48 and 72 h of incubation at 27°C

PCR analysis

Molecular identification of *E. amylovora* strains was carried out by amplification of 900 bp fragment of pEA29 in conventional PCR (Bereswill et al., 1992), and 391 bp fragment in nested PCR (Llop et al., 2000). DNA extraction was performed by a method of Pastrik & Maiss (2000). In both PCR reactions, the reference strains NCPPB 595 and CFBP 1430 were used as positive controls. PCR reactions were conducted in a Thermo Cycler 2720 (Applied Biosystem, USA). PCR products were separated by 1.5 % agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer, stained in ethidium

bromide (1 μg/ml) and visualized under UV light by a digital imaging camera (Vilber Lourmat, France).

Rep-PCR

Genetic diversity of the strains was analysed by using REP1R-1/REP2-1, ERIC1R/ERIC2 and BOXA1R primers in REP, ERIC and BOX-PCR, respectively (Versalovic et al., 1991; 1994). Reaction mixture of the final volume of 25 μl contained: 1 · PCR Master mix (Thermo Scientific, Vilnius, Lithuania), 4 μg BSA, 10 % DMSO, 3 μM REP1R-1/REP2-1, ERIC1R/ERIC2 and BOXA1R primers and 1 μl of DNA sample. PCR was carried out according to the following programme: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 94°C for 3 s and at 92°C for 30 s, annealing at 40°C (REP1R-1/REP2-1) or at 50°C (ERIC1R/ERIC2 and BOXA1R) for 1 min, and extension at 65°C for 8 min; and the final extension at 65°C for 8 min. Amplified PCR products were resolved by 2 % agarose gel electrophoresis in TAE buffer, stained and visualized as previously described.

RAPD-PCR

RAPD-PCR was carried out according to Momol et al., (1997), using six different primers (CUGEA1-CUGEA6) in independent reactions. Reaction mixture

Table 1. Bacterial strains used in tests

Bacterial strain	Host	Location	Year of isolation	Amplified fragment size (bp)		RAPD-PCR groups
				Conventional PCR (Bereswill et al., 1992)	Nested PCR (Llop et al., 2000)	
[a]KBI 30	Quince	Žiljci	2013	920	390	A
KBI 32	Quince	Žiljci	2013	930	400	C
KBI 33	Quince	Žiljci	2013	940	400	C
KBI 34	Quince	Žiljci	2013	920	400	C
KBI 37	Quince	Barajevo	2013	930	400	D
KBI 48	Quince	Valjevo	2013	960	440	D
[b]KFB 28	Quince	Petrovčić	2012	970	420	B
KFB 29	Quince	Petrovčić	2012	960	390	B
KFB 547	Quince	Zrenjanin	2012	1010	400	C
KFB 553	Quince	Medveđa	2012	980	390	C
KFB 557	Quince	Gola Glava	2012	990	400	C
KFB 559	Quince	Gola Glava	2012	1030	420	C
KFB 572	Quince	Vinča	2012	1040	410	B
KBI 52	Pear	Valjevo	2013	960	430	D
KBI 63	Pear	Lipolist	2014	960	440	C
KBI 66	Pear	Lipolist	2014	940	420	D
KBI 68	Pear	Predvorica	2014	950	410	C
KBI 69	Pear	Predvorica	2014	960	410	C
KBI 71	Pear	Predvorica	2007	950	430	C
KFB 562	Pear	Topola	2012	1030	400	D
KFB 568	Pear	Vinča	2012	1030	400	B
KFB 21	Apple	Mala Krsna	2007	970	450	B
KFB 22	Apple	Mala Krsna	2007	970	440	B
KFB 23	Apple	Mala Krsna	2007	960	440	B
KFB 27	Apple	Petrovčić	2007	970	460	B
KFB 681	Apple	Donji Miokovci	2013	1100	510	B
KFB 682	Apple	Donji Miokovci	2013	1100	510	B
KFB 683	Apple	Donji Miokovci	2013	1100	510	D
NCPPB 595	Pear	UK	1958	1060	410	B
CFBP 1430	*Crataegus* sp.	France	1972	1020	400	B

[a]KBI- collection of bacteria, Institute for Plant Protection and Environment, Belgrade, Serbia;
[b]KFB - collection of phytopathogenic bacteria, University of Belgrade, Faculty of Agriculture, Belgrade, Serbia

of the final volume of 20 µl contained: 1 · Dream Taq green buffer with 2 mM $MgCl_2$ (Thermo Scientific, Vilnius, Lithuania), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.45 µM CUGEA1-CUGEA6 primer, 0.8 U Taq DNA polymerase (Kapa Biosystems, USA) and 2 µl of DNA sample. The reaction was carried out according to the following program: initial denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 2 min; and final extension at 72°C for 5 min.

Amplified PCR products were resolved by 1.5 % agarose gel electrophoresis in TAE buffer, stained and visualized as previously described. The obtained genetic profiles were converted into a binary matrix with the recorded presence (1) or absence (0) of each amplified fragment, assuming that fragments of the same size in different bands were homologous. Phylogenetic analysis was carried out by using the FreeTree program (Hampl et al., 2001) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), using Nei-Li similarity coefficients (Nei & Li, 1979). Statistical significance was tested with 1000 "bootstrap" repetitions. A dendrogram was created using the TreeView program (Page, 1996).

RESULTS

Characteristics of E. amylovora *strains*

Both conventional and molecular tests confirmed that all studied strains possess characteristics typical for *E. amylovora*. Pathogenicity of the strains was confirmed on immature fruits of pear, cherry and plum, which showed discolouration of tissue and oozing at the point of inoculation after three days. All strains induced hypersensitive reaction on tobacco leaves 24 h after inoculation.

The strains were Gram-negative, levan-positive, oxidase-negative and catalase-positive, facultative anaerobes that hydrolyzed gelatine and did not produce fluorescent pigment on KB medium (Table 2). They utilized citrates, produced acid from sorbitol and reducing substances from sucrose, but were not able to reduce nitrates. All of them grew in the presence of 5 % NaCl, and not at 36°C. On NSA, the strains formed characteristic, large, shiny, noticeably convex and whitish, typical levan-type colonies after three days. On KB medium, they formed whitish, slightly convex colonies with even edges, 1-2 mm in diameter.

PCR reaction with primers A and B resulted in amplification of DNA fragments of 920-1100 bp, while DNA fragments of 390-510 bp were amplified by using the nested PCR method (Table 1).

Table 2. Biochemical and physiological characteristics of the studied strains

Test	Results[a]		
	Studied strains	Reference strains	
		NCPPB 595	CFBP 1430
Gram reaction	-	-	-
Fluorescence on King's medium B	-	-	-
Levan production	+	+	+
Oxidase activity	-	-	-
Catalase activity	+	+	+
Citrate utilization	+	+	+
Glucose metabolism	OF	OF	OF
Acid production from sorbitol	+	+	+
Growth at 36 °C	-	-	-
Growth in 5% NaCl	+	+	+
Nitrate reduction	-	-	-
Gelatin liquefaction	+	+	+
Reducing substances from sucrose	+	+	+
Hypersensitivity on tobacco	+	+	+
Pathogenicity assay			
Pear	+	+	+
Cherry	+	+	+
Plum	+	+	+

[a] + positive reaction; - negative reaction; OF - oxidative-fermentative metabolism of glucose

Genetic diversity of E. amylovora *strains*

DNA fingerprints obtained with the rep PCR did not show any differences among the studied *E. amylovora* strains. Genetic profiles obtained with REP primers were in a range approximately from 600 to 4000 bp, for ERIC primers they ranged between 150 to 2500 bp, and for BOX primers the amplified product ranged from 200 to 3000 bp.

On the other hand, the use of RAPD-PCR resulted in different genetic profiles of the studied strains (Figure 1).

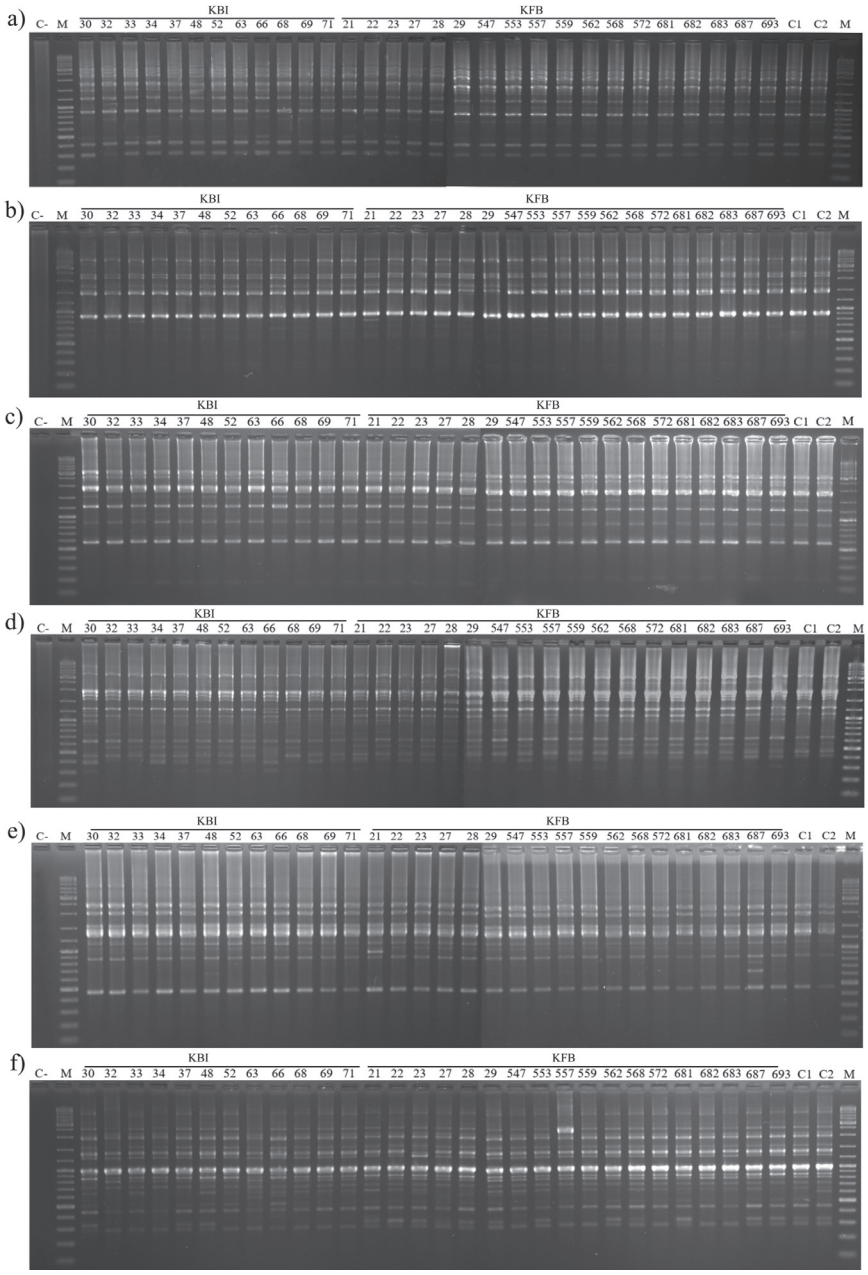

Figure 1. RAPD profiles of the studied *E. amylovora* strains generated with primers CUGEA1 (a), CUGEA2 (b), CUGEA3 (c), CUGEA4 (d), CUGEA5 (e) and CUGEA6 (f). Strain designations are indicated above the respective lanes. Lane M – molecular size marker GeneRuler DNA Ladder Mix; C1 – reference strain NCPPB 595; C2 – reference strain CFBP 1430.

Several genetic groups were differentiated based on the number and size of amplified DNA fragments by each primer, except CUGEA2. In RAPD-PCR with the primer CUGEA2 the obtained genetic profiles were identical for all studied strains. Among six primers, CUGEA4, CUGEA5 and CUGEA6 exhibited higher specificity than CUGEA1 and CUGEA3 primers. The use of primer CUGEA1 resulted in two genetic profiles, of which the most frequent one contained 13 highly repetitive fragments. This genetic profile was found in 28 strains, which was 93% of the total number (Figure 1a). Two different RAPD profiles were obtained by using the primer CUGEA3, of which the most frequent one contained seven highly repetitive fragments. This genetic profile was found in 25 strains, which was 83% of the total number (Figure 1c). The use of the primer CUGEA5 resulted in four genetic profiles, of which the most frequent one contained eight highly repetitive fragments. This profile was present in 27 strains, which is 93% of the total number (Figure 1e). Amplification of a larger number of DNA fragments of different sizes with the primers CUGEA4 and CUGEA6 complicated their grouping (Figure 1d and 1f).

A dendrogram generated by cluster analysis using six RAPD primers (CUGEA1, CUGEA2, CUGEA3, CUGEA4, CUGEA5 and CUGEA6), showed that 32 *E. amylovora* strains were differentiated into four genetic groups, marked with letters A-D (Fig. 2). Group B was the most numerous, consisting of 14 strains from different

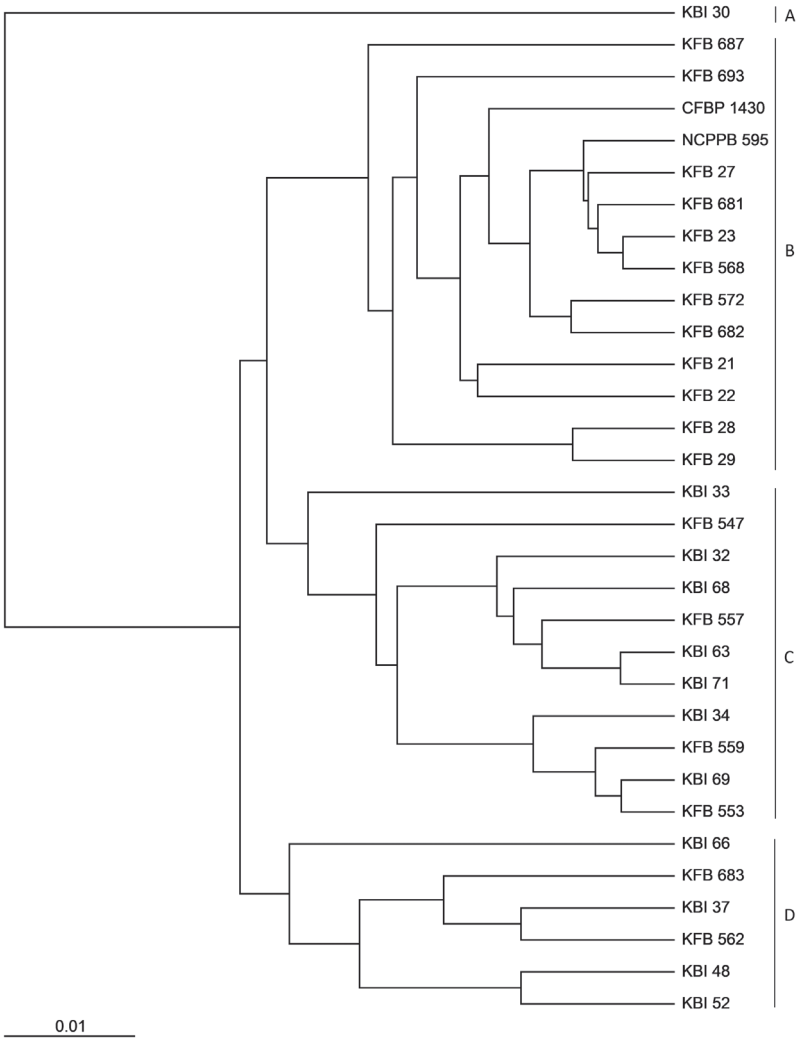

Figure 2. Similarity dendrogram based on UPGMA cluster analysis using the average Nei-Li similarity coefficients of the combined CUGEA 1, 2, 3, 4, 5 and 6 RAPD profiles of studied *E. amylovora* strains

locations, including two reference strains. Groups C and D consisted of 11 and six strains, respectively, while the group A consisted of one strain isolated from quince in 2013.

The obtained dendrogram could not show the relationship between RAPD results and host species. For example, out of six strains that were indistinguishable from each other by CUGEA 1–6 RAPD analyses, one strain (KFB 683) originated from apple, three from pear (KBI 66, KFB 562 and KBI 52) and two from quince (strains KBI 37 and KBI 48). In another cluster of 11 indistinguishable strains, four originated from pear (KBI 63, KBI 68, KBI 69 and KBI 71) and seven from quince (KBI 32, KBI 33, KBI 34, KFB 547, KFB 553, KFB 557 and KFB 559). The dendrogram also showed that there was no relationship between the RAPD results and the year of isolation (Figure 2). Among the 30 *E. amylovora* strains studied, 5 were isolated in 2007, 9 strains in 2012, 12 in 2013 and 4 strains in 2014. Also, the dendrogram showed that strains from the same year did not cluster together. For example, cluster C in the dendrogram (Figure 2) contains 11 *E. amylovora* strains isolated in 2007, 2012, 2013 and 2014.

DISCUSSION

The results of our research showed a genetic diversity among *E. amylovora* strains from different hosts and locations in Serbia, based on RAPD analysis. Application of CUGEA5 primer resulted in four genetic profiles, while two different RAPD profiles were obtained by using the primers CUGEA1 and CUGEA3. Other primers (CUGEA 4 and CUGEA6) amplified a large number of DNA fragments of different sizes which made their grouping difficult.

The results of standard and differential tests showed that all 30 strains were homogeneous regarding the biochemical and physiological characteristics typical for *E. amylovora*. Although all studied strains caused typical symptoms on immature fruits, there were slight differences in necrosis intensity. Different degrees of virulence of *E. amylovora* strains in the same plant genotype have been also reported by other authors (Hevesi et al., 2000; Sholberg et al., 2001; Pulawska et al., 2006). Pathogenicity of bacteria is generally determined by their capacity for biosynthesis of exopolysaccharides, which is the main component of bacterial exudate. Its production correlates with the virulence of a strain (Pulawska & Sobiczewski, 2012). Since necrosis and bacterial exudate occurred on inoculated pear fruits, but also on cherry and plum fruits, 72 h after inoculation, those fruits could be considered as suitable for pathogenicity test. However, symptom development on cherry and plum fruits indicates that those plants could be potential hosts of *E. amylovora*. Moreover, fire blight on European plum was recorded in Germany in 2002 (Vanneste et al., 2002) and in Hungary in 2011 (Végh et al., 2012).

Most strains used in this study were highly homogeneous regarding growth characteristics on KB and NSA media. Although a majority of them formed small, slightly convex and whitish colonies on KB medium, five strains (KBI 30, KBI 33, KBI 37, KBI 48 and KBI 52) formed mucoid colonies. Some differences in growth of the strains were also observed on NSA medium. Although all studied strains formed levan-type colonies, differences were observed in their size, convexity and edges, which grouped the strains into three types. Similar variations in the shape of colonies were also reported by Momol & Aldwinckle (2000), especially between strains isolated from *Maloideae* and *Rubus* spp., and later observed also by Ivanović et al., (2012). The results of our research did not indicate a correlation between the morphology of colonies on KB and NSA media and the pathogenicity of the strains, nor their host species or geographical origin.

The identity of all *E. amylovora* strains used in this study was confirmed by PCR with A and B primers (Bereswill et al., 1992). Product sizes ranging from 920-1100 bp were detected after amplification. Other authors have also reported that this primer set amplified fragments longer than 900 bp (Brown et al., 1996; Lecomte et al., 1997). The amplified fragments obtained by Lecomte et al., (1997) among 127 *E. amylovora* strains from all over the world, ranged between 900 and 1100 bp as well. Using the restriction analysis, they determined that different sizes of amplified fragments were caused by mutations in the nucleotide sequence of the plasmid pEA29. Jones & Geider (2001) emphasized that differences in the size of amplified fragments were caused by the number of short sequence repeats (SSR) of eight base pairs. The use of nested PCR resulted in amplification of DNA fragments of 390-510 bp. Variations in the size of amplified DNA fragments were also reported by Llop et al., (2000). Schnabel & Jones (1998) reported that differences in amplified fragments were caused by the SSR of eight base pairs, which could be found in different isolates in 3-15 copies.

Over the last two decades, there have been several attempts to differentiate *E. amylovora* strains worldwide. Rep PCR was one of the first techniques

for the assessment of genetic diversity of *E. amylovora* (McManus & Jones, 1995). In our research, this technique coupled with REP, BOX and ERIC primers did not result in differentiation of the studied strains. Several DNA fragments were amplified, with number and size identical in all 30 strains, including two reference strains. However, by rep-PCR, McManus & Jones (1995) observed slight differences in genetic profiles, especially between *Rubus* sp. strains and those isolated from pome fruits. For more than 170 strains, mostly from North America, only 2-3 genetic profiles were obtained using these primers, of which ERIC primers provided maximum variation.

The RAPD-PCR technique revealed differences in genetic profiles of the studied *E. amylovora* strains. Among the six primers used, CUGEA4, CUGEA5 and CUGEA6 were the most discriminant, each producing at least four different RAPD profiles (Figure 1). However, a phylogenetic analysis of combined data for the primers CUGEA1-CUGEA6 resulted in differentiation of a total of four different genetic groups (Figure 2). Analysis of the obtained genetic profiles by RAPD primers showed no correlation between the studied strains and their geographical locations, year of isolation and/or their hosts. On the other hand, Radunović et al., (2017) were able to differentiate strains from pear from other host-related strains by rep-PCR and also by RAPD-PCR (primers CUGEA 3 and 5) in a study of *E. amylovora* population from Montenegro. Further studies are necessary to elucidate this theory, as well as other aspects of host or location-specific grouping of *E. amylovora* strains. In addition, this is the first report of RAPD grouping of *E. amylovora* strains from Serbia.

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Karakterizacija i diverzitet populacije sojeva *Erwinia amylovora* poreklom iz jabučastih voćaka gajenih u Srbiji

REZIME

Primenom standardnih bakterioloških i molekularnih metoda proučen je diverzitet 30 sojeva *Erwinia amylovora* izolovanih iz dunje, kruške i jabuke, poreklom iz 14 lokaliteta u Srbiji. Svi proučavani sojevi izazvali su nekrozu i pojavu bakterijskog eksudata na nesazrelim plodovima kruške, trešnje i šljive, kao i hipersenzitivnu reakciju duvana. Proučavani sojevi bili su Gram i oksidaza negativni, fakultativno anaerobni, levan i katalaza pozitivni i nisu stvarali fluorescentni pigment na Kingovoj podlozi B. Svi sojevi hidrolizuju želatin, koriste citrate i stvaraju kiselinu iz sorbitola, proizvode redukujuće supstance iz saharoze, ne redukuju nitrate, razvijaju se u prisustvu 5% NaCl, ali ne i pri 36°C. Identitet sojeva potvrđen je konvencionalnim PCR i nested PCR metodama. Rep-PCR metodom korišćenjem REP, ERIC i BOX prajmera umnoženo je više fragmenata DNK čiji broj i veličina su se podudarali kod svih proučavanih sojeva. Za razliku od Rep-PCR, primenom RAPD-PCR metode uz korišćenje šest prajmera došlo je do izdavajanja različitih genetičkih profila i diferencijacije sojeva u četiri grupe. Genetičke razlike među proučavanim sojevima nisu bile u korelaciji sa domaćinima iz kojih su izolovani, niti sa njihovim geografskim poreklom i godinom izolacije.

Ključne reči: Bakteriozna plamenjača; Heterogenost; Patogenost; RAPD-PCR; Rep-PCR; Jabučaste voćke