MOLECULAR CHARACTERIZATION OF ERWINIA AMYLOVORA STRAINS ORIGINATED FROM POME FRUITS AND INDIGENOUS PLANT IN MONTENEGRO

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SUMMARY

In the period from 2012-2015 plant samples with fireblight symptoms were collected from pome fruits and indigenous plant, in main fruitgrowing regions of Montenegro. After successful isolation, pathogenicity of the obtained strains was tested by artificial inoculation of immature pear fruits, variety Viljamovka. Hypersensitive reaction was tested on tobacco leaves, variety White Burley. Identification and genetic diversity studies were performed using several molecular techniques on 18 Erwinia amylovora strains originating from quince, pear, apple and hawthorn. Bacterial identity was confirmed by nested PCR in which all studied strains produced the expected amplification fragment of plasmid pEA29. To detect potential genetic variations in E. amylovora population, rep-PCR was conducted. Using REP, ERIC and BOX primers, in all three PCR reactions, differences between studied strains were detected, i.e. pear strains had different genetic profiles from all other studied strains, including reference strain. Genetic variability of selected E. amylovora strains was studied by RAPD-PCR as well. Both of the used random primers, CUGEA-3 and CUGEA-5, showed discriminatory potential by separating genetic profiles of pear strains from all other studied strains, including reference strain. This is the first study of genetic variability of E. amylovora in Montenegro.

Keywords: fireblight, rep-PCR, RAPD-PCR, population diversity.

INTRODUCTION

Erwinia amylovora (Burrill) Winslow is the causal agent of fireblight, one of the most destructive diseases of fruits and ornamental plants (van der Zwet and Beer, 1999). In South Europe and in the Balkan E. amylovora occurred during the eighties of the last century and rapidly spread in almost all Mediterranean countries. On the territory of ex Yugoslav countries, bacterium was first detected in Macedonia on pear in 1989 (Panić and Arsenijević, 1996), then in 1990 in Serbia (Panić and Arsenijević, 1996; Gavrilović and Arsenijević, 1998) and Bosnia and Herzegovina (Arsenijević et al., 1991), in Croatia in 1995 (Crjete-ković et al., 1999), and the latest in Slovenia in 2001-2002 (Dreo et al., 2006).

In Montenegro, fireblight symptoms were observed for the first time in 1996 on pear trees in vicinity of Bijelo Polje, in northern part of the country (Arsenijević and Gavrilović, 2007). The occurrence was experimentally confirmed in 2003 on apple samples from vicinity of Nikšić (Obradović et al., 2003).

More intensive studies of E. amylovora in Montenegro conducted in the period from 2012 to 2015 confirmed that the bacterium is widely spread in the whole country, particularly in fruit growing regions in northern, continental part. Presence of the bacterium was confirmed on four fruit species: quince (Cydonia oblonga), pear (Pyrus communis), apple (Malus domestica), medlar (Mespilus germanica) including one species from spontaneous flora, hawthorn (Crataegus sp.) (Balaž et al., 2012; Radunović and Gavrilović, 2013). Studied isolates of the bacterium originating from different localities and different hosts showed pronounced uniformity in pathogenic, cultural, morphological and biochemical-physiological characteristics (Radunović et al., 2013). Serological analysis confirmed high homogeneity in antigenic structure of the studied strains of E. amylovora originating from quince, pear, apple and hawthorn from different localities in Montenegro (Radunović et al., 2015a).

Monitoring of the effect of meteorological factors on fireblight symptom occurrence in different regions of Montenegro revealed their high correlation. Meteorological conditions in bloom phase of the susceptible fruit species in northeastern (localities Bijelo Polje and Berane) and
western (locality Nikšić) parts of the country favor fire- light infections. Quince, pear and apple orchards, as well as single heavily infected trees in the mentioned regions, are hotspots from which the bacterium spreads to other areas and new hosts (Radunović et al., 2015b).

Presented data point to the fact that in the last few years E. amylovora spread in continental part of the country where it caused significant damages. Therefore, this bacterium is a serious threat to successful cultivation of pome fruits, especially because areas under pome fruits are becoming larger each year in Montenegro (Radunović et al., 2013).

Considering significance of studies of E. amylovora natural populations and lack of this data in Montenegro, this research aimed at identification and determination of genetic differences between E. amylovora strains from quince, pear, apple and hawthorn, from different localities in Montenegro. Several molecular techniques were applied: Nested PCR (using two primer pairs AJ75, AJ76, PEANT1 and PEANT2), Rep-PCR (using REP, ERIC and BOX primers) and RAPD PCR (using two primers CUGEA3 and CUGEA5).

### MATERIALS AND METHODS

**Isolation of bacteria and pathogenicity tests.** The bacteria were isolated from collected plant parts (branches, leaves, fruits and flowers) expressing fireblight symptoms by standard bacteriological techniques on NSA and King’s B media (KB) (Lelliott and Stead, 1987; Arsenijević, 1997; Schaad et al., 2001). Bacterial cultures were kept on nutrient agar medium with 2% of glycerol (NAG), at 4°C in refrigerator. Bacterial cultures grown on KB, 24 h old were used in all assays (Arsenijević, 1997; Schaad et al., 2001; Jones and Geider, 2001).

Pathogenicity of the obtained isolates was tested by inoculation of immature pear fruits, variety Viljamovka, using bacterial suspension (concentration of $10^8$ CFU/ml). Inoculated fruits were put in plastic boxes and incubated under conditions of high moisture at 25°C. Development of fruit necrosis with bacterial exudate was observed after 3-5 days and was considered as positive reaction. Fruit inoculated with sterile, distilled water were used as a negative control, and reference strain NCPPB 595 (National Collection of Plant Pathogenic Bacteria - UK) as positive control (Lelliott and Stead, 1987, Klement et al., 1990).

**Molecular analyses.** Molecular analyses were performed on 18 strains originating from quince, pear and apple growing near hawthorn, from different localities in Montenegro. Studied strains of E. amylovora are shown in Table 1.

**Bacterial DNA extraction.** Bacterial cultures of E. amylovora grown 24 h on KB medium at 27°C (Llop et al., 2000) were used. Bacterial suspension at a concentration of $10^8$ CFU/ml was prepared in sterile, distilled water. DNA extraction was performed by cell lysis in water bath at 95°C for 10 min. Microtubes with bacterial DNA were cooled on ice, centrifuged and used for analyses.

**Nested PCR.** For confirmation of the identity of the studied strains, nested PCR with insert-specific primers was applied (Llop et al., 2000), with specific primers for detecting matching sequence of 391 bp on plasmid pEA29. Two primer pairs were used simultaneously in one PCR tube (Table 2).

PCR mix of total volume of 50 μl was prepared as follows: 31.8 μl of molecular grade water; 1× PCR buffer; 3 mM MgCl$_2$; 2 μl formamide; 0.2 mM dNTPs; 0.03 pmol primer AJ75 and AJ76; 10 pmol primer PEANT1 and PEANT2; 3 U Taq polymerase and 1 μl of DNA sample.

PCR reaction was performed in Thermal Cycler (Applied Biosystems 2720, USA) under the following conditions: first round of amplification consisted of an initial denaturation at 94°C for 4 min and 25 cycles of 94°C for 30 s and 72°C for 1 min. In the same thermocycler, after initial denaturation at 94°C for 4 min, the second round of amplification consisted of 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 45 s. Final extension was at 72°C for 10 min.

Analysis and visualization of amplified PCR products was conducted after electrophoresis in 1.5% agarose gel (BluePower500 SERVA Electrophoresis GmbH) and ethidium-bromide staining. Visualization was performed

### Table 1. E. amylovora strains used in molecular analyses.

<table>
<thead>
<tr>
<th>Number</th>
<th>Strain code</th>
<th>Host plant</th>
<th>Cultivar</th>
<th>Plant part</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EaM 1</td>
<td>Cydonia oblonga</td>
<td>Leskovacka</td>
<td>branch</td>
<td>Bijelo Polje</td>
</tr>
<tr>
<td>2</td>
<td>EaM 2</td>
<td>C. oblonga</td>
<td>Leskovacka</td>
<td>branch</td>
<td>Berane</td>
</tr>
<tr>
<td>3</td>
<td>EaM 3</td>
<td>C. oblonga</td>
<td>Leskovacka</td>
<td>branch</td>
<td>Berane</td>
</tr>
<tr>
<td>4</td>
<td>EaM 4</td>
<td>C. oblonga</td>
<td>Leskovacka</td>
<td>leaf</td>
<td>Berane</td>
</tr>
<tr>
<td>5</td>
<td>EaM 5</td>
<td>C. oblonga</td>
<td>Leskovacka</td>
<td>leaf</td>
<td>Berane</td>
</tr>
<tr>
<td>6</td>
<td>EaM 6</td>
<td>C. oblonga</td>
<td>Leskovacka</td>
<td>leaf</td>
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</tr>
<tr>
<td>7</td>
<td>EaM 7</td>
<td>Malus domestica</td>
<td>Koza*a</td>
<td>branch</td>
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</tr>
<tr>
<td>8</td>
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<td>Jonathan</td>
<td>branch</td>
<td>B.Polje</td>
</tr>
<tr>
<td>9</td>
<td>EaM 9</td>
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<td>Iadar</td>
<td>branch</td>
<td>Nikšić</td>
</tr>
<tr>
<td>10</td>
<td>EaM 10</td>
<td>M. domestica</td>
<td>Budimka*</td>
<td>branch</td>
<td>Nikšić</td>
</tr>
<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>EaM 12</td>
<td>Crataegus sp.</td>
<td>/</td>
<td>branch</td>
<td>B.Polje</td>
</tr>
<tr>
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<td>Crataegus sp.</td>
<td>/</td>
<td>branch</td>
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</tr>
<tr>
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<td>EaM 14</td>
<td>Crataegus sp.</td>
<td>/</td>
<td>branch</td>
<td>Berane</td>
</tr>
<tr>
<td>15</td>
<td>EaM 15</td>
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<td>William's</td>
<td>branch</td>
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</tr>
<tr>
<td>16</td>
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<td>P. communis</td>
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</tr>
<tr>
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</tr>
<tr>
<td>18</td>
<td>EaM 18</td>
<td>P. communis</td>
<td>Junsko Zlato</td>
<td>branch</td>
<td>Berane</td>
</tr>
</tbody>
</table>

*a autochthonous cultivar
in UV transilluminator (Vilber Lourmat, Marne la Vallée Cedex, France).

Rep PCR. Repetitive sequence-based PCR (Rep PCR) was used to detect genetic variations among studied *E. amylovora* strains. REP (repetitive extragenic palindromic), ERIC (Enterobacterial Repetitive Intergenic Consensus) and BOX (Box elements) primers were used to detect presence of short repetitive sequences in bacterial genome (Versalovic *et al*., 1991, 1994; Louws *et al*., 1994) (Table 3). Amplification of unique parts present between repetitive segments in bacterial DNA enables separation of strains by profiles.

Three separate PCR reactions with suitable primers were performed (final volume of PCR mixture 25 µl). PCR mix for REP and ERIC PCR consisted of 1.3 µl molecular grade water; 12.5 µl of 2X PCR Master Mix (Fermentas, Lithuania); 0.2 µl of BSA (20 mg/ml); 2.5 µl of DMSO; 3.75 µl of primer Rep1R-1 i Rep2-1 (20 µM) or ERIC1R i ERIC2 (20 µM). PCR mix for BOX PCR consisted of 5.05 µl molecular grade water; 12.5 µl of 2× PCR Mix; 0.2 µl of BSA (20 mg/ml); 2.5 µl of DMSO; 3.75 µl of primer BOXAIR (20 µM).

Reaction conditions were as follows: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min and elongation at 72°C for 2 min. Final extension was at 72°C for 5 minutes.

After 1.5% agarose gel electrophoresis and ethidium-bromide staining, the profiles were observed under UV light.

### RESULTS

**Bacterial strains and pathogenicity tests.** Isolation of bacteria from infected plant parts on NSA medium resulted in formation of convex, creamy-colored, shiny, mucoid, “levan” type colonies characteristic for *E. amylovora*. On KB medium, small, white, circular colonies without fluorescent pigment were observed.

All studied strains caused necrosis of inoculated pear fruits with occurrence of bacterial exudate three days after inoculation. Tobacco leaf necrosis as a sign of hypersensitive reaction occurred 24 h after infiltration of bacterial suspension.

**Molecular analyses.** Nested PCR. In nested-PCR, a specific DNA fragment was amplified for all 18 studied strains (Fig. 1). For six studied strains, EaM 2, EaM 3, EaM 15, EaM 16, EaM 17 and EaM 18, and for the reference strain NCPPB 595, fragments of the expected size of 391 bp were amplified.
amplified. Fragments sized 447 bp were amplified for five tested strains, EaM 1, EaM 7, EaM 8, EaM 9 and EaM 10, while for seven strains, EaM 4, EaM 5, EaM 6, EaM 11, EaM 12, EaM 13 and EaM 14, fragments of 416 bp were obtained. Rep PCR. Genetic profiles of the studied strains were obtained by REP, ERIC and BOX primers. Strains from pear formed one profile type while strains from quince, apple and hawthorn formed another type, i.e. pear strains had different genetic profiles from all other studied strains, including reference strain. All pear strains (EaM 15-EaM 18), regardless locality, had 8 identical fragments sized 400 to 4000 bp. All quince, apple and hawthorn strains (EaM 1-EaM 14), as well as in positive control, 4 identical fragments sized 1200 to 3000 bp were observed (Fig. 5).

Strains from pear amplified 5 identical profiles in range from 700 to 1500 bp when CUGEASE primer was used, while strains from quince, apple and hawthorn amplified 7 identical profiles in range from 500 to 5000 bp. Discrete polymorphism was observed in one strain from quince (EaM 1) in which a fragment sized 1000 bp was amplified. The fragment was not present in other strains from quince, apple and hawthorn. Reference strain had similar genetic profile with strains from quince, apple and hawthorn, with the absence of two fragments, one sized 850 bp and the other sized 1100 bp (Fig. 6).

DISCUSSION

This is the first study of genetic variability of *E. amylovora* in Montenegro. Applied molecular methods showed high specificity and sensitivity in identification and differentiation of 18 studied strains from pear, quince, apple and hawthorn, from different localities. Nested PCR enabled use of two primer pairs in two simultaneous PCR reactions in a single PCR tube. This resulted in higher sensitivity, shorter detection time and reduced contamination risk, which classifies this method among routine methods for *E. amylovora* detection in plant material (Llop et al., 2000). In this study, DNA fragments of all studied strains were amplified as a single band of the expected size with nested PCR, which confirmed their identity as *E. amylovora*. Small variations detected between the strains i.e. difference in 56 nucleotides in size of amplified fragments (from 391 to 447 bp) can be explained by repetitive sequence of 8 bp (GAATTACA), which can vary in different strains (Schnabel and Jones, 1998; Llop et al., 2000).

Rep PCR is one of the first molecular techniques used not only for diagnostic purposes, but also for epidemiological studies of *E. amylovora* (McManus and Jones, 1995b; Versalović et al., 1991). McManus and Jones (1995b) easily detected differences between strains from *Rubus* and *Pomoideae* plant species by this method. Using Rep-PCR,
Pulawska and Sobiczewski (2012) separated 2-3 genetic profiles among strains from North America, and the most pronounced variations were obtained by ERIC primers. Using Rep PCR Barionovi et al. (2006) showed that majority (89 of 93 strains) of E. amylovora strains from Maloideae and Rosoideae, from different regions, had the same DNA profile, while diversity was ascertained for strain isolated from Amelanchier sp. (Maloideae) grown in Canada and strains from Rubus sp. In our study, REP, ERIC and BOX primers detected differences between strains from pear and all other studied strains therefore, therefore this method is suitable for differentiation of genetic profiles in the studied E. amylovora population in Montenegro.

Among different molecular techniques, RAPD-PCR is very useful and simple for fast differentiation of strains (Pržulj and Perović, 2005). Compared to other markers, RAPD markers do not require knowing genomic sequences. Each primer enables amplification of several different points in the genome. This makes RAPD markers suitable for detection of polymorphisms between individual strains (Tingey et al., 1993) and for studies of species genetic diversity (Baum et al., 1997). Momol et al. (1997) applied this method in studying 16 E. amylovora strains from USA, Europe and Japan. Differences in genomes of the studied strains were detected using 6 random primers CUGEA1-CUGEA6, and based on the obtained RAPD profiles, all strains were classified into three groups. However, in RAPD analysis with 5 random primers, Pulawska et al. (2006) confirmed high homogeneity of 14 E. amylovora strains, originating from 6 hosts from different regions in Poland. In our study, RAPD analysis with 2 random primers, was suitable for studying heterogeneity of E. amylovora population considering that, as with Rep PCR, genetic differences between tested strains could be detected and pear strains were again separated from other studied strains by this method.

Although numerous literature data point to E. amylopora as homogeneous species (Billing et al., 1961; Paulin, 2000; Pulawska et al., 2006; Pulawska and Sobiczewski, 2012), certain molecular techniques enabled detection of differences between strains originating from different hosts and geographical areas (McManus and Jones, 1995a; Kim et al., 1996; Momol and Aldwinckle, 2000; Brennan et al., 2002; Obrodović et al., 2007; Ivanović et al., 2010, 2012). Molecular methods enabled significantly faster identification of the bacterium compared to conventional techniques (Bereswill et al., 1992), as well as differentiation of strains within the species. Knowledge on genetic diversity is particularly important for epidemiological studies and for determination of spreading direction, virulence and pesticide and antibiotic resistance in the bacterium (Paulin, 2000; Jones and Schnabel, 2000; Janse, 2006).

In our study, all applied molecular techniques enabled fast identification of the bacterium and detection of differences in genetic profiles of E. amylovora strains, separating strains from pear from other strains originating from different hosts. Detected polymorphism in strains from pear is probably the consequence of adaptation to host, i.e. of changes that occurred in the genome and caused formation of new points to which primer was attached (Williams et al., 1990; Jones et al., 1997). It is interesting that all strains from pear, regardless locality, had identical profiles. Also, all other studied strains originating from different localities and hosts (quince, apple and hawthorn) had identical profiles. No differences between strains originating from different localities were detected in this study.

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