



Isolation and characterization of *Pseudomonas syringae* isolates affecting stone fruits and almond in Montenegro

Tamara Popović¹  · Jelena Menković²  · Anđelka Prokić²  · Nevena Zlatković³  · Aleksa Obradović² 

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Abstract

In Montenegro, stone fruit species are grown on intensive and semi-intensive commercial plantations. However, almond production is mainly organized on family gardens and for household consumption. During two seasons (2017–2018), we surveyed apricot, peach, nectarine, sweet cherry, Japanese plum, and almond orchards for the presence of bacterial diseases at different geographical locations in Montenegro. From leaf, petiole and fruit lesions, branch or twig cankers, and necrotizing buds, a total of 29 isolates were obtained and subjected to identification based on their morphological, pathogenic, biochemical, and molecular characteristics. Pathogenicity of the isolates was confirmed by reproducing the symptoms on leaves, fruits, and twigs of the corresponding host plants. The biochemical tests indicated that the isolates belong to *Pseudomonas syringae*. However, isolates' characterization showed variation in their phenotypic and molecular features. The presence of the *syrB* gene and ice nucleation activity grouped most of the isolates within pathovar *syringae*. The results of rep-PCR using the BOX primer revealed high genetic diversity of isolates. Multilocus sequence analysis (MLSA), using four housekeeping genes, showed that 27 isolates belong to the genomic species 1, *P. syringae* sensu stricto, corresponding to *P. syringae* phylogroup 2. However, isolates from the same phylogroup 2 did not form a monophyletic group. One strain isolated from apricot was most distinct and similar to members of genomic species 2, phylogroup 3. All tested isolates showed significant levels of resistance to copper sulfate and high level of sensitivity to streptomycin sulfate in vitro.

Keywords Stone fruits · Almond · *Pseudomonas syringae* · Identification · MLSA · Copper resistance

Introduction

The genus *Pseudomonas* is one of the most complexes in bacterial taxonomy. The *Pseudomonas syringae* phylogenetic group comprises 15 recognized bacterial species closely related to *P. syringae* and more than 60 pathovars (Gomila et al. 2017). Although the taxonomy of the *P. syringae* species complex has been a relevant topic over the last 40 years, it still remains a controversial group of bacteria (Gutiérrez-Barranquero et al. 2019). The classification is defined based on the host range and symptomatology,

dividing *P. syringae* species into pathogenic varieties (Young et al. 1978; Dye et al. 1980; Young 2010). Furthermore, within pathovars, isolates may be distinguished into races that show specificity toward particular host cultivars (Dye et al. 1980; Joardar et al. 2005; Young 2010; Hulin et al. 2018). Based on DNA–DNA hybridization analysis of the *Pseudomonas syringae* species, complex nine genomospecies were established (Gardan et al. 1999). Multilocus sequence typing (MLST) based on similarity of housekeeping genes had a significant impact on the *P. syringae* phylogenetic classification (Bull et al. 2011; Parkinson et al. 2011; Berge et al. 2014) allowing delineation of thirteen phylogenetic groups (PGs) within the complex. A recent study of this species using comparative genomics suggested that a high number of strains were misclassified (Gomila et al. 2017).

Pseudomonas syringae is a globally important plant pathogen, causing different diseases in more than 180 host plant species (Bultreys and Kałużna 2010). Several pathovars within the *P. syringae* species complex are known to

✉ Tamara Popović
tamara.popovic@ubh.gov.me

¹ Administration of Food Safety, Veterinary and Phytosanitary Affairs, Podgorica, Montenegro

² University of Belgrade, Faculty of Agriculture, Belgrade, Serbia

³ Institute for Plant Protection and Environment, Belgrade, Serbia

cause diseases on plants belonging to *Prunus* spp. (Young 1987; Kamiunten et al. 2000; Ménard et al. 2003; Kaluzna et al. 2016): these pathovars are distributed throughout PG1, PG2 and PG3 of the *P. syringae* species complex (Ruinelli et al. 2019).

P. syringae pathovars *syringae*, *morsprunorum* and *persicae* are the most harmful pathogens of stone fruits and almond trees causing significant damage in nurseries and orchards, and reducing the fruit quality and yield worldwide (Young 1987; Kennelly et al. 2007; Sulikowska and Sobiczewski 2008; Spotts et al. 2010; Scortichini 2010). In the European area, *P. syringae* pathovars and races were confirmed to cause bacterial canker in stone fruit orchards in Germany (Hinrichs-Berger 2004), Belgium (Gilbert et al. 2009), Poland (Sulikowska and Sobiczewski 2008; Bultreys and Kałużna 2010; Kaluzna et al. 2010), Serbia (Balaž et al. 2016), UK (Hulin et al. 2018), and Italy (Giovanardi et al. 2018).

Due to the phenotypic and genomic heterogeneity of *P. syringae* pathovars, a multiphasic approach is necessary to identify these bacteria (López et al. 2010). Since conventional tests are not sufficient to differentiate isolates at the pathovar level, similarities and differences between the pathovars can be determined either by toxin production, genetic profiles (rep-PCR), multilocus sequence analysis (MLSA) and other molecular methods (Versalovic et al. 1991; Little et al. 1998; Sorensen et al. 1998; Schaad et al. 2001; Hwang et al. 2005; Gilbert et al. 2009).

Bacterial canker of sour cherry trees (*P. cerasus* L.) was previously reported in Montenegro (Vučinić et al. 1992): the disease was found in young orchards on more than 50% of the trees from 1983 to 1987. The sensitivity of the sour cherry cultivars, as well as favorable weather conditions, contributed to a rapid spread of the disease. Based on pathogenicity, morphological and some biochemical–physiological properties, it was concluded that the sour cherry isolates belong to *P. syringae* (Vučinić et al. 1992).

During a 2-year survey, different symptoms: branch or twig cankers, leaf, petiole and fruit lesions, and necrotizing buds were observed on various stone fruit species and almond, in Montenegro. The economic importance of the disease and lack of effective control measures prompted etiological studies and identification of the causal agent. The aim of this study was to characterize bacterial strains preliminary attributed to *P. syringae*, originating from different hosts and geographical regions in Montenegro. Therefore, we studied phenotypic and molecular properties of a total of 29 *Pseudomonas* strains isolated from stone fruits (apricot, peach, nectarine, sweet cherry, and Japanese plum) and almond.

Materials and methods

Plant material and sampling

During the growing seasons 2017 and 2018, stone fruit and almond trees, grown in commercial orchards and home gardens in Montenegro, were surveyed for the presence of symptoms referring to possible bacterial diseases. Symptomatic plant material (branch or twig cankers, leaves, petioles and fruits with lesions, and necrotic buds) was collected from early spring until the beginning of summer (more precisely, from March until the end of June).

Isolation and purification of bacterial isolates

Symptomatic plant samples were rinsed with tap water, surface disinfected with 70% ethanol, and air-dried. Small fragments were taken from the border area between apparently healthy and diseased tissue, and macerated with pestle and mortar in 1 ml of sterile distilled water (SDW). The homogenate was streaked onto nutrient agar (NA) plates and incubated at 27 °C for 48 h. Single colonies, morphologically resembling *P. syringae*, were picked and re-streaked on the same medium to ensure purity (Lelliott and Stead 1987). A total of 29 bacterial isolates obtained from different stone fruit species (apricot, peach, nectarine, sweet cherry, and Japanese plum) and almond were characterized in this study (Table 1). The pathotype strain of *P. syringae* pv. *syringae* (Pss, KFB¹ 0103 = NCPPB² 281, isolated in 1950 from *Syringa vulgaris*) was used as a reference. The isolates were stored in nutrient broth (NB) supplemented with 30% glycerol at – 80 °C. Prior to testing, bacterial cultures were grown on NA plates at 27 °C for 24 h, unless indicated differently.

Pathogenicity tests

The pathogenicity of isolates was tested in laboratory conditions by inoculating shoots, leaves, and immature fruits of the corresponding host plant species the isolates were originating from. For artificial inoculation, bacterial suspensions were prepared in sterile distilled water from 24-h-old cultures grown on NA medium at 27 °C. The inoculum concentration was adjusted to approx. 10⁸ CFU/ml. Three inoculation methods were used: spraying of young shoots by a hand-held sprayer, prick inoculation of immature fruits leaving a drop of the inoculum in the lesion, and leaf infiltration

¹ KFB, Collection of Phytopathogenic Bacteria, University of Belgrade, Faculty of Agriculture, Serbia.

² NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, Sand Hutton, York, UK.

Table 1 Bacterial isolates used in this study

| Isolate | Host plant | Isolated from | Location | Date of isolation | GenBank Accession numbers | | | | |
|---------|---------------|---------------|-------------|-------------------|---------------------------|-------------|-------------|-------------|----------|
| | | | | | <i>gyrB</i> | <i>rpoD</i> | <i>gapA</i> | <i>gltA</i> | 16R rRNA |
| K1 | Apricot | Bud | Bar | March 2017 | MT265365 | MT250587 | MT295219 | MT345301 | MT355196 |
| K2 | Apricot | Bud | Bar | March 2017 | MT265366 | MT250588 | MT295220 | MT345302 | MT355159 |
| K3 | Apricot | Branch canker | Bar | March 2017 | MT265367 | MT250589 | MT295221 | MT345303 | |
| JŠ1 | Japanese plum | Twig canker | Ulcinj | March 2017 | MT265378 | MT250600 | MT295232 | MT345314 | |
| BR1 | Peach | Twig canker | Podgorica | May 2017 | MT265375 | MT250597 | MT295229 | MT345311 | |
| K4 | Apricot | Fruit | Podgorica | May 2017 | MT265368 | MT250590 | MT295222 | MT345304 | |
| T1 | Sweet cherry | Leaf | Bar | May 2017 | MT265374 | MT250596 | MT295228 | MT345310 | |
| K5 | Apricot | Leaf | Danilovgrad | June 2017 | MT265369 | MT250591 | MT295223 | MT345305 | |
| K6 | Apricot | Branch canker | Danilovgrad | March 2018 | MT265370 | MT250592 | MT295224 | MT345306 | |
| B1 | Almond | Leaf | Tuzi | April 2018 | MT259031 | MT212403 | MT295206 | MT314427 | |
| B2 | Almond | Leaf | Tuzi | April 2018 | / | / | / | / | MT355441 |
| K7 | Apricot | Twig canker | Podgorica | April 2018 | MT265371 | MT250593 | MT295225 | MT345307 | |
| K8 | Apricot | Twig canker | Bar | April 2018 | MT265372 | MT250594 | MT295226 | MT345308 | |
| BR2 | Peach | Twig canker | Kotor | April 2018 | MT265376 | MT250598 | MT295230 | MT345312 | |
| K9 | Apricot | Petiole | Podgorica | May 2018 | MT265373 | MT250595 | MT295227 | MT345309 | |
| N1 | Nectarine | Fruit | Tuzi | May 2018 | MT265377 | MT250599 | MT295231 | MT345313 | |
| B3 | Almond | Fruit | Zeta | May 2018 | MT259032 | MT240937 | MT295207 | MT314428 | |
| B4 | Almond | Leaf | Zeta | May 2018 | MT259033 | MT240938 | MT295208 | MT314429 | |
| JŠ2 | Japanese plum | Twig canker | Bar | May 2018 | MT265379 | MT250601 | MT295233 | MT345315 | |
| B5 | Almond | Leaf | Zeta | May 2018 | MT265355 | MT240939 | MT295209 | MT314430 | |
| B6 | Almond | Leaf | Zeta | May 2018 | MT265356 | MT240940 | MT295210 | MT314431 | |
| B7 | Almond | Leaf | Podgorica | June 2018 | MT265357 | MT240941 | MT295211 | MT345293 | |
| B8 | Almond | Twig canker | Podgorica | June 2018 | MT265358 | MT240942 | MT295212 | MT345294 | |
| B9 | Almond | Leaf | Podgorica | June 2018 | MT265359 | MT240943 | MT295213 | MT345295 | |
| B10 | Almond | Leaf | Budva | June 2018 | MT265360 | MT240944 | MT295214 | MT345296 | |
| B11 | Almond | Leaf | Budva | June 2018 | MT265361 | MT240945 | MT295215 | MT345297 | |
| B12 | Almond | Leaf | Budva | June 2018 | MT265362 | MT240946 | MT295216 | MT345298 | |
| B13 | Almond | Leaf | Nikšić | June 2018 | MT265363 | MT240947 | MT295217 | MT345299 | |
| B14 | Almond | Leaf | Nikšić | June 2018 | MT265364 | MT240948 | MT295218 | MT345300 | MT355438 |

of the bacterial suspension from the abaxial surface using a syringe without needle, in three replicates, respectively. SDW and the pathovar reference strain KFB 0103 were used as negative and positive controls, respectively. Inoculated leaves and fruits were maintained on a moist sterile filter paper in sterile Petri dishes, while the shoots were kept with their cut end dipped in flasks containing tap water (vol. 250 ml). The dishes with the inoculated fruits and shoots were then placed into appropriate sealed plastic boxes to ensure high humidity. Symptom development was observed up to 12 days after inoculation. Koch's postulates for each pathogenic bacterial isolate were completed by re-isolation and identity check.

Phenotypic characterization

Prior to testing, the investigated isolates were grown on NA at 27 °C for 24 h. The following bacterial

characteristics were studied: Gram reaction, production of a green fluorescent pigment on King's medium B, catalase activity, oxidative-fermentative metabolism of glucose, vitality on nutrient sucrose agar (NSA) medium (Lelliott and Stead 1987); ice nucleation activity (Fahy and Hayward 1983); levan production on NSA, oxidase production, pectolytic activity, arginine dihydrolase activity, and hypersensitivity on tobacco leaves (LOPAT test; Lelliott et al. 1966); as well as gelatin liquefaction, aesculin hydrolysis, tyrosinase activity, and utilization of L-tartaric acid (GATTa tests; Lelliott and Stead 1987). All tests were carried out in two replicates. Reference strains of Pss (KFB 0103), *P. s. pv. persicae* (Psp—KFB 0102, NCPPB 2761), *P. s. pv. morsprunorum* (Psm race 1—KFB 0120, and race 2—KFB 0101, NCPPB 2995), were included as positive controls.

Table 2 PCR method and list of primers used in this study

| PCR method | Primer sequences (5′–3′) | Size of amplified product | References |
|-------------|---------------------------------------------------------------------|---------------------------|------------------------|
| <i>syxB</i> | B1: CTTCCGTGGTCTTGATGAGG B2: TCGATTTTGCCGTGATGAGTC | 752 bp | Sorensen et al. (1998) |
| <i>gapA</i> | gapAFp: CCGGCSGARCTGCCSTGG gapARps: GTGTGRTTGGCRTCGAARATCGA | ~ 650 bp | Hwang et al. (2005) |
| <i>gltA</i> | gltAFp: CCTCBTGGCAGTCGAAGATCACC gltARp: TTGTAVGGRCYGGAGAGCATTTC | ~ 1050 bp | Hwang et al. (2005) |
| <i>gyrB</i> | gyrBFps: TCBGCRGCVGARGTSATCATGAC gyrBRps: TTGTCTTGGTCTGSGAGCTGAA | ~ 750 bp | Hwang et al. (2005) |
| <i>rpoD</i> | rpoDFp: AGGTGGAAGACATCATCCGCATG rpoDRps: CCGATGTTGCCTTCTGGATCAG | ~ 1100 bp | Hwang et al. (2005) |
| 16S rRNA | fD1: AGAGTTTGATCCTGGCTCAG rP2: ACGGCTACCTTGTACGACTT | ~ 1450 bp | Weisburg et al. (1991) |
| BOX PCR | BoxA1R: CTACGGCAAGGCGACGCTGACG | / | Schaad et al. (2001) |

Genomic DNA extraction

Total genomic DNA was extracted from all selected isolates and reference strains using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Quality of extracted DNA was checked by gel electrophoresis on 0.8% agarose gel. The DNA samples were stored at -20°C until use.

Detection of the *syxB* gene

In order to detect *syxB* gene, the specific primers B1 and B2 (Table 2) were used to amplify the 752 bp DNA fragment (Sorensen et al. 1998). PCR reaction was performed in a Thermo Cycler 2720 (Applied Biosystems, USA) in a total volume of 25 μl . PCR master mix contained: 18.25 μl nuclease-free water, 2.5 μl of 10 \times Dream Taq Green Buffer (includes 20 mM MgCl_2) (Thermo Scientific, Vilnius, Lithuania), 0.5 μl of 10 mM dNTP mix, 1.25 μl of each 10 μM primer, 0.25 μl of 5 U/ μl Dream Taq DNA polymerase (Thermo Scientific, Vilnius, Lithuania), and 1 μl of the template DNA.

The PCR products (5 μl) were separated by agarose gel (1.5%) electrophoresis in Tris–acetate–EDTA (TAE) buffer at room temperature and 80 V for 45 min, stained in ethidium bromide (1 $\mu\text{g}/\text{ml}$) and visualized under UV light by a digital imaging camera (Vilber Lourmat, France). For negative control, template was replaced with the same volume of SDW. The DNA extract from the reference Pss strain was included as *syxB* positive control.

Molecular characterization

Genetic relatedness among the isolates was investigated by rep-PCR, using the BOX primer (Table 2) (Schaad et al. 2001). The reference strains Pss (KFB 0103), Psm race 1

(KFB 0120) and race 2 (KFB 0101), and Psp (KFB 0102), were used for comparison and SDW as a negative control.

The 16S rRNA sequence was analyzed for four *syxB* negative isolates, two isolated from apricot (K1 and K2), and two isolated from almond (B2 and B14). Amplicons for the partial 16S rRNA sequences, about 1.024 bp in length, were generated using the universal primers fD1/rP2 (Weisburg et al. 1991) (Table 2). Chromatograms obtained by sequencing (Macrogen Europe, The Netherlands) were analyzed using Finch TV 1.4.0 software. Sequences (“forward” and “reverse”) were processed using the MEGA X software (Kumar et al. 2018). The BLAST program (Altschul et al. 1997) was used for comparative analysis of the obtained sequences with the sequences deposited in the NCBI database. The partial sequences generated with 16S rRNA gene were deposited in the GenBank database (Table 1).

The MLSA phylogenetic analysis was performed by PCR amplification and partial sequencing of 4 constitutive genes: *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *gltA* (citrate synthase), *gyrB* (gyrase B) and *rpoD* (RNA polymerase sigma⁷⁰ factor) for 28 tested isolates according to the protocol of Hwang et al. (2005) (Table 2). Minor adjustment of the PCR protocol was made by increasing the annealing temperature by 5 $^{\circ}\text{C}$ for the amplification of the *rpoD* gene. Due to the low sequence quality obtained for the *rpoD* gene sequence, strain B2 was not included in the MLSA analysis.

Gene sequences (Macrogen Europe, The Netherlands) were assembled and edited using the FINCH TV v.1.4.0 software. For comparative analysis of investigated sequences, the BLAST program was used (Altschul et al. 1997). The partial sequences of the four housekeeping genes (*gapA*, *gltA*, *gyrB* and *rpoD*) were deposited in the GenBank database (Table 1). Multiple alignments and comparisons with reference strains available in the NCBI database and the Plant Associated Microbes Database (PAMDB) were

performed using CLUSTAL W algorithm (Higgins et al. 1996) integrated into MEGA X software (Kumar et al. 2018).

Phylogenetic analysis of isolates was performed by the Maximum Likelihood method, with genetic distance between sequences calculated according to the Kimura-2 model (Kimura 1980). Phylogenetic trees were constructed using the individual alignments of *rpoD* (498 nt), *gapA* (468 nt), *gltA* (528 nt), and *gyrB* (507 nt) (not shown) and a concatenated data set. A bootstrap analysis of 1,000 replications was performed.

Copper sulfate and streptomycin sensitivity assay in vitro

We studied copper sulfate and streptomycin sensitivity in vitro of the 29 isolates, as well as control strains Pss (KFB 0103), Psm race 1 (KFB 0120), Psm race 2 (KFB 0101) and *Xanthomonas arboricola* pv. *pruni* (Xap—KFB 0146, NCPPB 416).

The bacterial culture, grown on NA for 48 h, was suspended in sterile distilled water and concentrated to approx. 1×10^8 CFU/ml. The suspension droplets of each isolate (3 μ l) were spotted on the surface of sucrose peptone agar (SPA) plates (Lelliott and Stead 1987), amended with CuSO_4 (100, 200 ppm) or streptomycin sulfate (25, 50 ppm). *Xanthomonas euvesicatoria*, strain E-3 (KFB 062), resistant to these compounds was used as a control. SPA plates without amendments were used as a blank control. The plates were incubated at 28 °C for 48 h and observed for bacterial growth. Isolates able to grow at given concentrations of the bactericide were scored as resistant. All tests were repeated in two replicates.

Results

Isolation and purification of bacteria

During the 2017–2018 survey of stone fruits commercial orchards and home gardens in Montenegro, symptoms resembling those caused by pathogenic bacteria were observed.

Severe leaf-spotting was noticed on almond trees, affecting more than 80% of the canopy. The leaf spots were numerous, round, necrotic, light to dark brown, varying in size, usually surrounded by a halo. They were located irregularly, sometimes all over the leaf blade, but sometimes along the leaf edge or central midrib. In some cases, necrosis and yellowing started from the leaf tip and spread toward the petiole, causing leaf drop and defoliation eventually. However, on apricot and sweet cherry leaves, small necrotic spots, usually surrounded by a weak halo,

appeared sporadically. The diseased leaf samples resulted in 15 successful isolations, 12 isolates from almond, two from apricot leaves and petiole, and one from sweet cherry leaves.

Unlike on leaves, more severe symptoms were observed on apricot twigs and branches. Very often buds did not open in the spring. They were dark brown, necrotic, surrounded with sunken lesion. Two isolates were isolated from apricot bud samples. Additionally, dark, elongated, depressed canker wounds, usually associated with gummosis, were formed along the apricot twigs and branches. Similar cankers were also observed on Japanese plum, peach and almond twigs, and branches. Nine positive isolations indicated that the canker symptoms were associated with putative pathogenic bacteria, four from apricot, two from Japanese plum and peach, respectively, and one from the almond sample.

Symptoms on fruits also varied depending on the host plant: on apricot fruits flat, superficial, brown spots were observed, while on nectarine and almond fruits lesions were sunken, brown, and scaly, associated with gummosis. Three bacterial isolates were isolated from the diseased apricot, nectarine, and almond fruits, and they were included in this study.

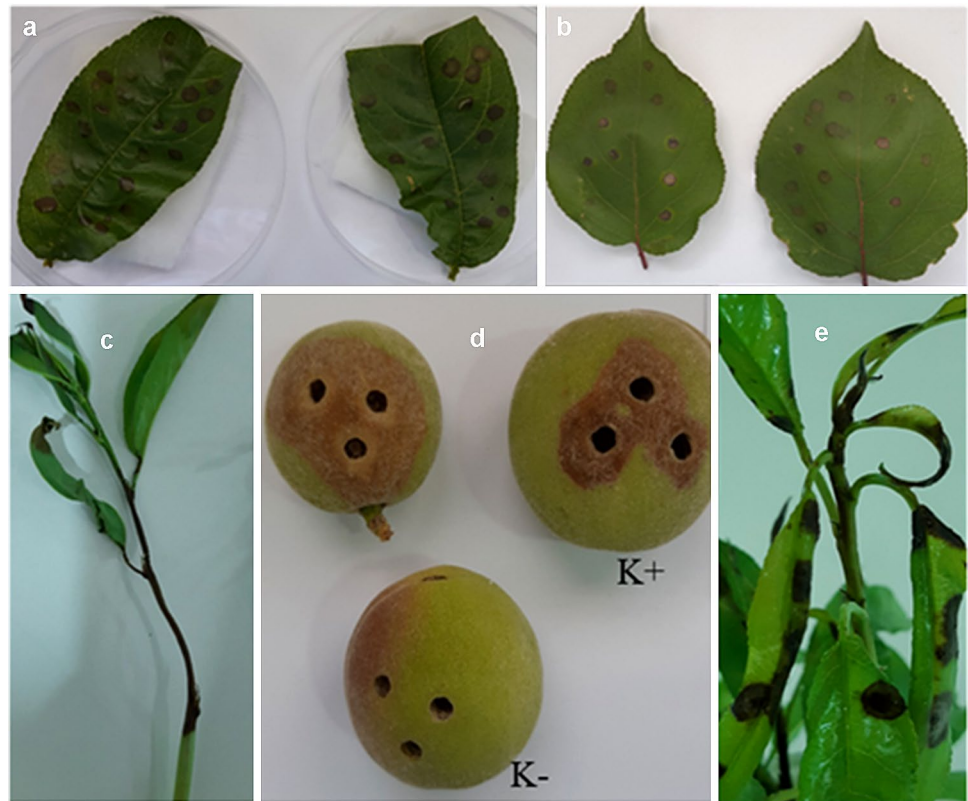
Generally, the most intense symptoms were observed on almond leaves and apricot branches and twigs, causing defoliation and, in some cases, complete plant death.

Small (2 mm in diameter), circular, glossy, flat, entire edge grayish-white colonies predominated on NA plates after 2–3 days of incubation at 27 °C. Representative colonies were re-streaked for purity checking. Strain B7 produced a brown pigment in the medium.

Pathogenicity tests

Pathogenicity of isolates was confirmed by reproducing the symptoms on leaves, fruits, and twigs of their corresponding host plants (Fig. 1). On inoculated leaves, water soaking areas developed around the inoculation site within 3–6 days. Later, lesions became necrotic. Similar symptoms were observed on the inoculated immature fruits, 2–10 days after inoculation, depending on the host. Tissue around the fruit inoculation sites became necrotic and sunken, resulting in lesions development and merging on some fruits after 5–7 days. Wilting of the inoculated shoots was noticed 7 days after inoculation, followed by spread of the brown-black necrosis from the inoculation point 5 days later. Reference Pss strain KFB 0103, used as a positive control in pathogenicity tests, also caused symptoms described above on inoculated shoots, leaves and fruits, while no symptoms were observed on tissue inoculated with SDW.

Fig. 1 Pathogenicity test: **a** necrotic spots on *Prunus persica* leaves caused by isolate N1, 6 days after inoculation; **b** necrotic spots on *P. armeniaca* leaves caused by isolate K9, 6 days after inoculation; **c** shoot necrosis on *P. persica* caused by isolate BR1, 12 days after inoculation; **d** necrosis of fruits of *P. persica* caused by isolate N1, 6 days after inoculation; **e** shoot necrosis on *P. dulcis*, 12 days after inoculation; K+ positive control Pss (KFB 0103); K– negative control



Phenotypic characterization

All isolates were Gram-negative, obligate aerobic, catalase positive, and produced fluorescent pigment on KB medium. The results of levan production differentiated isolates into two groups. Fifteen isolates were clearly levan-positive, and according to LOPAT scheme, they belong to the *Pseudomonas* Group Ia. The remaining 14 isolates did not clearly produce levan, while the rest of the characteristics corresponded to the *Pseudomonas* Group Ia. The results of GATTa tests differentiated 5 isolates and corresponding control strain as Pss, while the rest of the investigated isolates showed inconclusive results (Table 3). All tested isolates hydrolyzed aesculin, while gelatin hydrolysis, tyrosinase activity, and utilization of L-tartrate varied. However, neither of them matched with the control Psm or Psp strains. On NSA medium, all isolates remained vital up to 4 days and 21 even after 7 days. Twenty-five isolates showed INA at -4°C , while four isolates were negative (Table 3).

Detection of the *syrB* gene

PCR with B1 and B2 primers detected *syrB* gene by amplifying a product of 752 bp in 25 isolates and reference Pss strain (Fig. 2).

Differentiation by Rep-PCR

The genomic DNA fingerprints of 29 isolates were determined using BOX-PCR. The obtained profiles showed genetic variability among the isolates (Fig. 3). Out of all tested isolates 12 profiles could be differentiated. Some of them were represented by a single strain (BR2, B10, B14, K6) while some had up to four strains with similar profiles clustering together (JS1, K5, B1, B2; B3, B4, B11, B12; K3, K8, B5, B6). They were not host specific, comprising isolates obtained from different host species. None of the profiles matched entirely with the control strains. However, most of them shared several bands in common with the Pss (KFB0103) control strain (Fig. 3). The genetic fingerprints did not correspond to the isolates' origin either.

16S rRNA sequence analysis

The 16S rRNA gene was analyzed for those four strains, for which the gene for syringomycin synthesis was not detected. The comparison of sequences with those previously deposited in the NCBI database showed that they belong to Pss. The 16S rRNA gene sequence of K1 and K2 strains showed 100% and B14 more than 99% identity to Pss strain 176, isolated from apricot in Iran (GeneBank accession number KY569214). The 16S rRNA gene sequence of B2 showed

Table 3 Biochemical and physiological characteristics of the studied isolates

| Isolate | Gram staining | Fluorescent pigment on KB | L O P A T | G A T Ta | O/F test | Catalase activity | Vital-ity (after 7 days) | INA activity (-4 °C) | Copper resistance (200 ppm) | Streptomycin resistance (2.5 ppm) | syxB |
|-----------------|---------------|---------------------------|-----------|----------|----------|-------------------|--------------------------|----------------------|-----------------------------|-----------------------------------|------|
| K3 | - | + | + | + | O | + | - | + | + | - | + |
| BR1 | - | + | + | + | O | + | + | + | + | - | + |
| B1 | - | + | + | + | O | + | + | + | + | - | + |
| B2 | - | + | + | + | O | + | + | + | + | - | - |
| B8 | - | + | + | + | O | + | + | + | + | - | + |
| JS2 | - | + | + | + | O | + | + | + | + | - | + |
| K8 | - | + | + | + | O | + | + | + | + | - | + |
| BR2 | - | + | + | + | O | + | + | + | + | - | + |
| N1 | - | + | + | + | O | + | + | + | + | - | + |
| B5 | - | + | + | + | O | + | + | + | + | - | + |
| B6 | - | + | + | + | O | + | + | + | + | - | + |
| B7 | - | + | + | + | O | + | + | + | + | - | + |
| B10 | - | + | + | + | O | + | + | + | + | - | + |
| B13 | - | + | + | + | O | + | + | + | + | - | + |
| B14 | - | + | + | + | O | + | + | - | + | - | - |
| K7 | - | + | + | + | O | + | + | + | + | - | + |
| B12 | - | + | + | + | O | + | + | - | + | - | + |
| B3 | - | + | + | + | O | + | + | + | + | - | + |
| B4 | - | + | + | + | O | + | + | + | + | - | + |
| B9 | - | + | + | + | O | + | - | + | + | - | + |
| B11 | - | + | + | + | O | + | + | + | + | - | + |
| K4 | - | + | + | + | O | + | + | + | + | - | + |
| K1 | - | + | + | + | O | + | - | - | + | - | - |
| K2 | - | + | + | + | O | + | - | - | + | - | - |
| K6 | - | + | + | + | O | + | - | + | + | - | + |
| K5 | - | + | + | + | O | + | - | + | + | - | + |
| K9 | - | + | + | + | O | + | + | + | + | - | + |
| T1 | - | + | + | + | O | + | - | + | + | - | + |
| JS1 | - | + | + | + | O | + | - | + | + | - | + |
| Pss KFB 0103 | - | + | + | + | O | + | - | + | + | - | + |
| Psm R1 KFB 0120 | - | + | + | + | O | + | / | / | + | - | / |
| Psm R2 KFB 0101 | - | + | + | / | O | + | - | - | + | - | / |
| Psp KFB 0102 | - | - | + | - | O | + | - | - | + | - | / |

+ positive reaction; - negative reaction;/not tested; O oxidative metabolism

Fig. 2 PCR detection of *gyrB* gene in tested isolates. K—negative control, K+—positive control Pss (KFB 0103); M—MassRuler Low Range DNA Ladder, Fermentas, Lithuania

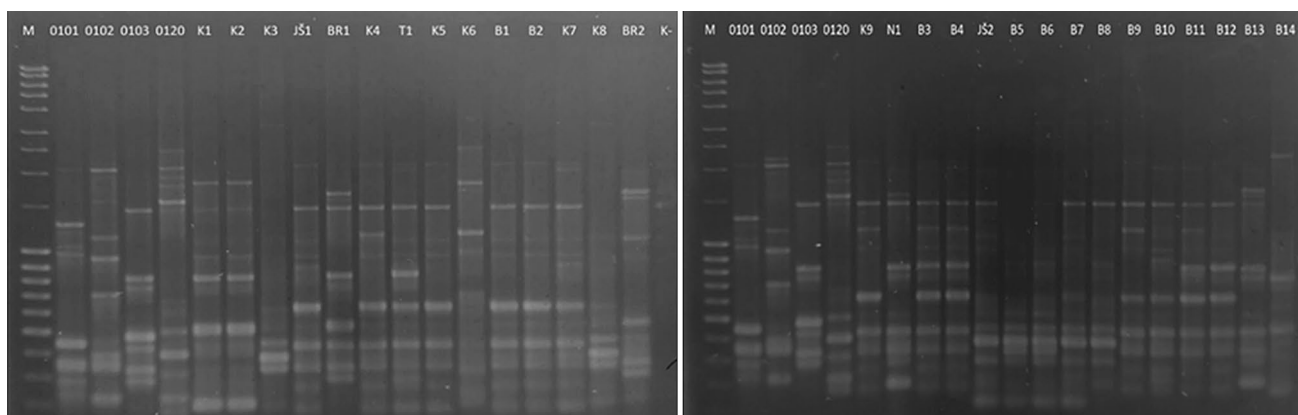
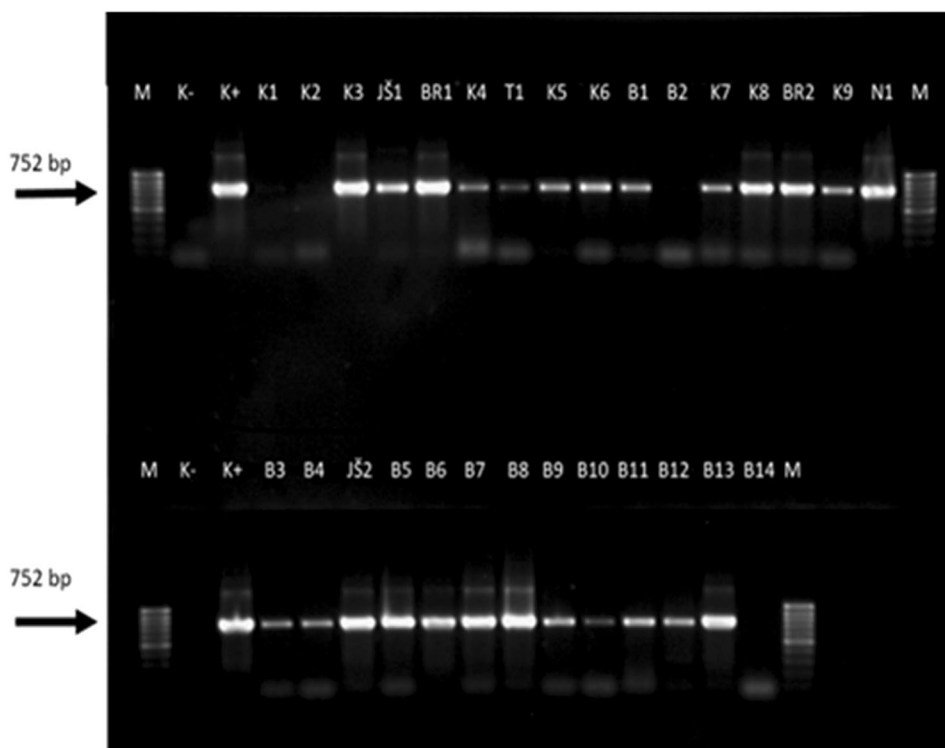


Fig. 3 BOX-PCR profiles generated with the BOX A1R primer. 0101, 0102, 0103, 0120—reference strains, isolates from different host plants (K—apricot, BR—peach, T—sweet cherry, N—nectarine, JS—Japa-

nese plum, B—almond), K—negative control, M—MassRuler DNA Ladder Mix, Fermentas, Lithuania

99.80% identity to Pss strains SHPS007 and SHPS008 isolated from apple in South Korea (GeneBank accession number KP753380 and KP713782). The partial sequences generated with 16S rRNA gene were deposited in the GenBank database (Table 1).

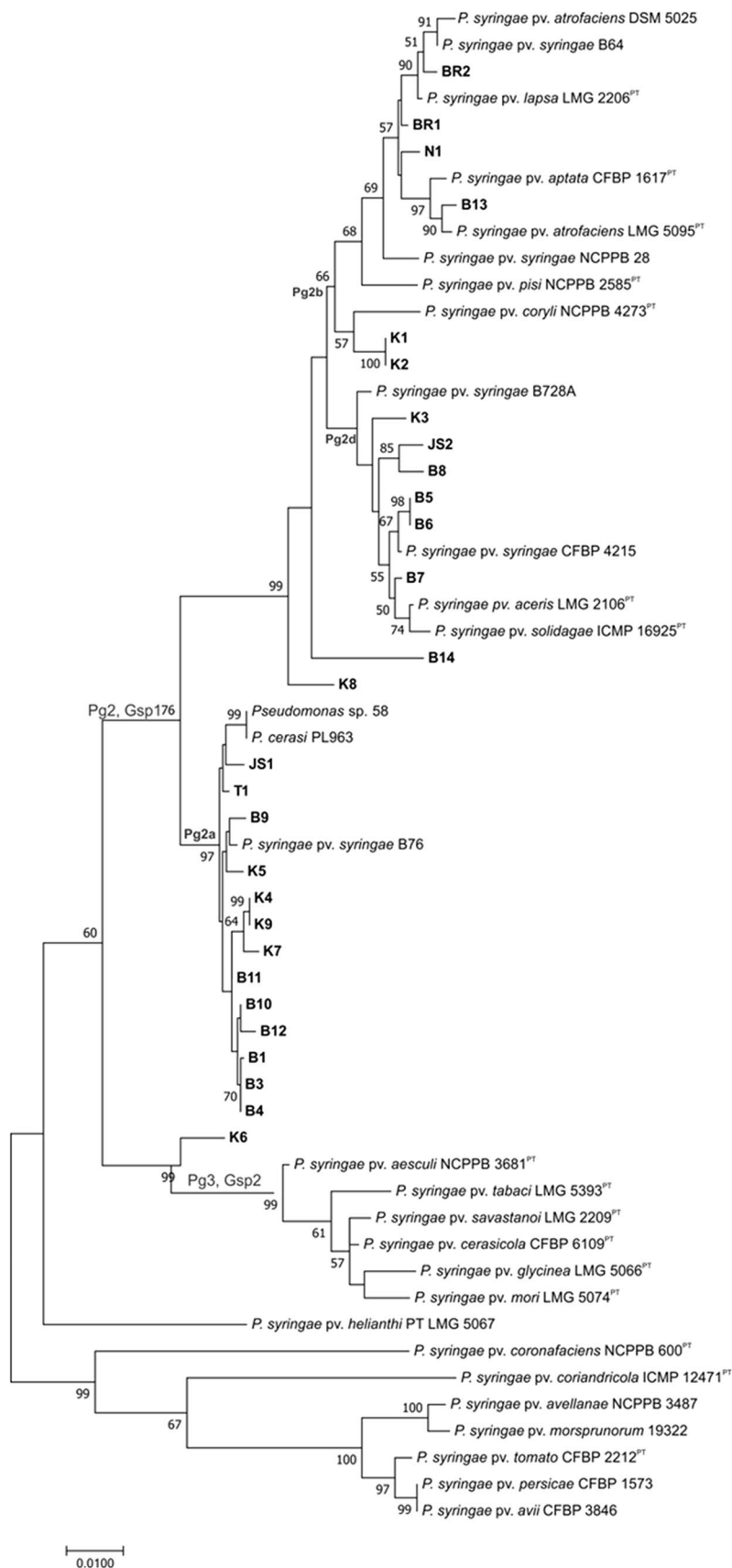
Multilocus sequence analysis (MLSA)

In order to analyze the genetic variability and clarify the phylogeny of 28 tested isolates, MLSA analysis based on 4 housekeeping genes (*gyrB*, *rpoD*, *gapA* and *gltA*) was

performed. The phylogenetic tree was generated based on the concatenated gene sequences consisting of a total length of 2001 nucleotides.

According to MLSA, most of the isolates, with the exception of K6, clustered within the genomospecies 1, *P. syringae* sensu stricto, among three separate clusters belonging to phylogroups (PG) 2a, 2b, and 2d (Fig. 4). Six strains (BR1, BR2, N1, B13, K1, and K2) were phylogenetically similar to the PG 2b while strains K3, JS2, B5, B6, B7 i B8 formed subcluster within PG 2d. These two PGs were closely related. Strains B14 and K8 differentiated from the

Fig. 4 Phylogenetic tree for the 28 bacterial isolates (in bold) constructed on the basis of MLSA concatenated sequences of four genes (*gapA*, *gltA*, *gyrB*, and *rpoD*), using Maximum Likelihood method, with genetic distance calculated according to the Kimura-2 model. The length of the branches corresponds to the rate of substitution of base pairs. The confidence of the nodes was estimated with 1,0000 bootstrap replicates shown at cluster nodes when > 50. Phylogroups are labeled PG2 (2a, 2b, 2d) and PG3, while the tested strains are bold. GenBank accession numbers are given in brackets



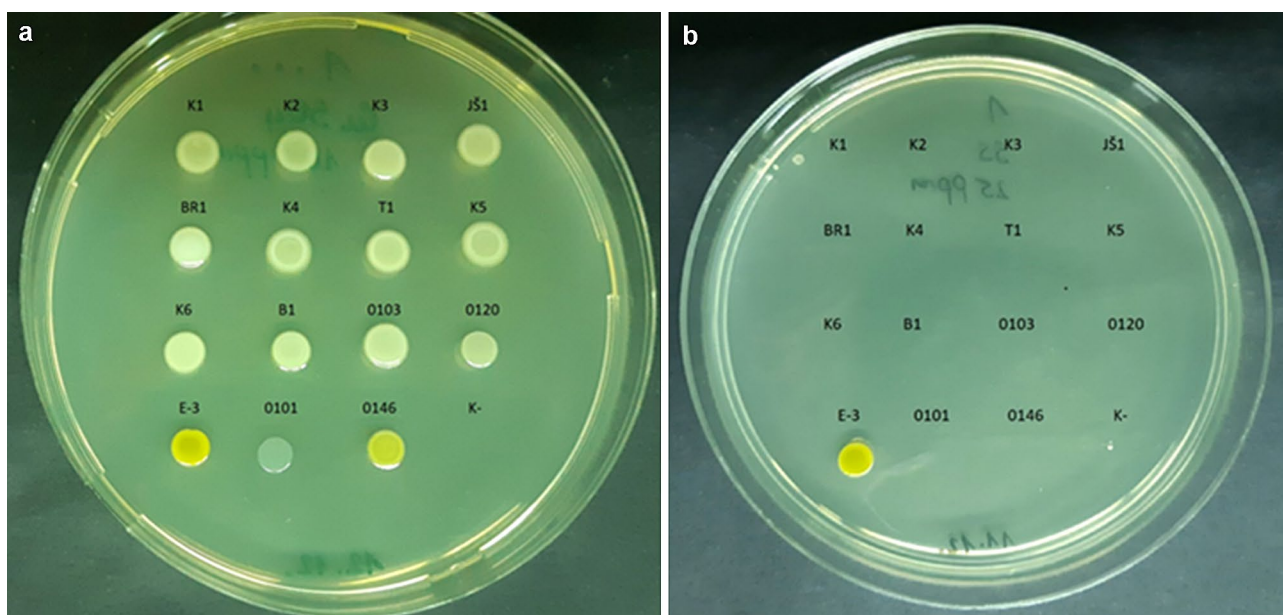


Fig. 5 Isolate development on SPA plates amended with: **a** 200 ppm of copper sulfate; **b** 25 ppm of streptomycin sulfate. E-3 positive control strain, K – negative control

remaining strains and placed in a separate subcluster most similar to PG 2d. Strains B1, B3, B4, B9, B10, B11, B12, K4, K5, K7, K9, T1, JS1 formed second subcluster together with two strains of *Pseudomonas cerasi* and *P. s. pv. syringae* strain B76 belonging to PG 2a. The strain K6 clustered separately since it showed the lowest similarity with other strains from this study, as well as the strains from the database. It was most closely related with the strains belonging to PG 3, genomospecies 2 (Fig. 4).

Copper sulfate and streptomycin sensitivity assay in vitro

All isolates grew on SPA plates amended with 100 ppm and 200 ppm of copper sulfate, indicating the presence of copper tolerance mechanisms. However, streptomycin sulfate inhibited growth of all studied isolates at the lowest concentration tested (25 ppm), with the exception of the resistant control strain *X. euvesicatoria* E-3 (Fig. 5).

Discussion

Fruit production provides considerable incomes for the growers in many countries worldwide. Stone fruits are extensively grown in most of the EU, although species distribution varies greatly among the Mediterranean and northern European countries (EFSA 2014). This production is particularly significant for Montenegro, where climate provides favorable growing conditions. In the fruit production

structure in Montenegro, plum is the most widespread stone fruit species, followed by peach (Preškić et al. 2016). In this area, stone fruits are grown on intensive and semi-intensive plantations. However, almond production is mainly organized on family gardens and for household consumption. Due to the extensive production and lack of efficient control, Montenegro stone fruit and almond production are often compromised by different phytopathogenic bacteria, especially when environmental conditions favor infection and spread. This population of pathogens was not studied in details recently. Apart from *Xanthomonas arboricola* pv. *pruni* detected in some peach samples (Popović et al. 2020), isolations indicated dominant presence of pseudomonads in the stone fruit samples collected during two growing seasons.

Xps, the causal agent of stone fruit bacterial canker, is one of the 60 pathogens that infects and causes economic losses in many plant species belonging to various families (Young 2010). The pathogen attacks different plant organs of stone fruits and almond causing cankers and necrosis of woody tissue, blight of buds and flowers, spots on the leaves and fruits, and wilting of twigs.

In this study, we characterized bacterial isolates obtained from apricot, peach, nectarine, sweet cherry, Japanese plum, and almond originating from different geographical locations in Montenegro. A total of 29 isolates were identified based on morphological, pathogenic, biochemical and molecular characteristics.

The isolates were isolated during early spring and summer from stone fruits in two subsequent growing seasons.

This part of the season was indicated as the most favorable for pathogen isolation (Giovanardi et al. 2018) since the multiplication and spreading of bacteria from active cankers present on fruit trees is the most intense in spring, reaching a maximum during flowering stage (Spotts et al. 2010). The pathogen was isolated from symptomatic plant material—leaf and fruit lesions, branch or twig cankers, and necrotizing buds and petioles. During the 2 year survey of orchards and almond trees, over 100 symptomatic samples were collected, but not all isolations yielded pathogenic bacteria, indicating different disease etiology. Therefore, symptoms could not be a reliable diagnostic criterion, since similar symptoms may be caused by other biotic or abiotic factors. Some fungi also affect stone fruits causing similar symptoms (e.g., *Wilsonomyces carpophilus*, formerly *Coryneum beijerinckii* causing shot-hole on leaves, twig blights, and bud necrosis): they may be confused with bacterial infections, leading to wrong orchard management. In order to differentiate pathogenic isolates, tobacco hypersensitivity was used as discriminatory test. All tested isolates induced HR in tobacco leaves and produced a fluorescent pigment on KB. Although growth on KB and production of green fluorescent pigment is a common differential characteristic of the genus *Pseudomonas*, this test can be indicative for the distinction of *Pss* and *Psm* from *Psp*.

In pathogenicity tests, we successfully reproduced the symptoms on inoculated host plant tissues. Obtained results showed that use of the detached organs (shoots, leaves, and fruits) instead of the whole plant is practical and provides reliable results. Additionally, the detached leaf inoculation test has some advantages: symptoms develop quickly, leaves could be used for a longer period than shoots and fruits, the method is rapid, reproducible and simple to perform (Moragrega et al. 2003; Bedford et al. 2003).

Growth and biochemical characteristics of our isolates indicated that they belong to the species *P. syringae*. Besides positive HR and fluorescence, all the isolates were Gram-negative, catalase positive, metabolized glucose oxidatively, and hydrolyzed aesculin.

Considering the growth on NA and KB plates, an exception was the strain B7, which produced a brown pigment in these media. Such ability of *Pseudomonas* sp. was reported by Kałużna et al. (2013) and Kałużna (2019) in isolates isolated from cornelian cherry (*Cornus mas*) and blueberry (*Vaccinium corymbosum*) in Poland. LOPAT tests have been routinely used to differentiate *P. syringae* from other fluorescent *Pseudomonas* species (Lelliott et al. 1966). However, some of the studied isolates did not clearly produce levan, therefore not entirely matching with the LOPAT scheme. Similarly, such behavior was also described for some *P. syringae* isolates obtained from stone fruits. They formed flat, no levan-type colonies, but still identified as *Pss* (Roos and Hattingh 1983; Gavrilović 2006).

P. syringae pathovars affecting stone fruits could be differentiated according to another set of differential tests, known as GATTa tests. The results indicated considerable variation among the isolates in these traits (Table 3). The majority of our isolates did not match exactly with the control *Pss* strain. Aesculin hydrolysis was the only characteristic the isolates had in common, while the other three isolates were variable, indicating limited differential value of GATTa tests in case of our isolates. In addition, the results from the GATTa tests for other *P. syringae* pathovars are still unknown, which may cause further complications in using these tests.

Gašić et al. (2012) reported that pathovar *morsprunorum* lost its vitality and showed negative catalase reaction after 4 days on NSA plates, while the pathovars *syringae* and *persicae* remained vital for at least seven days. Our isolates maintained their vitality longer than 4 days. Ice nucleation activity (INA) at -4°C also indicated close relatedness of the tested isolates to pv. *syringae*. Some strains of *P. syringae*, including pv. *syringae*, catalyze ice crystal formation on and in plant tissues (Lindow 1983). According to Roos and Hattingh (1983), the INA test is quite reliable in differentiating the pv. *morsprunorum* that shows no INA. However, these authors pointed out that some strains of pv. *syringae*, as well as intermediate strains, did not show any INA (Roos and Hattingh 1983).

Our experience supports earlier conclusions that biochemical tests are not sufficient and reliable for differentiating *P. syringae* strains at or below the pathovar level (Little et al. 1998). Biochemical tests, though useful for identification of bacterial genera and species, are often not discriminatory enough to determine *P. syringae* pathovars (Morris et al. 2000). Berge et al. (2014) reported that phenotypic characteristic provide only limited means for identification of isolates at the clade or phylogroup level and that phenotypes of isolates are variable among and within phylogroups. Pathovars of *P. syringae*, associated with stone fruits and nuts, produce several well-characterized phytotoxic compounds that can be used for pathovar differentiation. Syringomycin is a low molecular weight non-specific toxin, produced by *P. syringae* pvs. *aptata*, *atrofaciens*, and *syringae*. This phytotoxin is the main virulence factor of *Pss* and it is responsible for necrosis development in plant tissues: because of this trait, *Pss* is distinguished from *Psm* and *Psp*. Thus, detection of *syrB* gene was routinely used for determination of *Pss* strains (Sorensen et al. 1998; Scorticini et al. 2003; Gilbert et al. 2009; Kałużna et al. 2010). In our study, the *syrB* gene coding for syringomycin synthesis was detected in 25 tested isolates. Bultreys and Kałużna (2010) also reported the presence of *Pss* strains negative for syringomycin production. Interestingly, some *P. syringae* strains may lose this ability when stored for a long period (Hwang et al. 2005).

Our investigation of the *Pseudomonas* strains collection, by using classical techniques and conventional PCR, was followed by genomic fingerprinting using BOX-PCR and analysis of 16S rRNA gene, *gyrB*, *rpoD*, *gapA*, and *gltA* genes as phylogenetic markers, leading to the identification of different genomic groups. The results of rep-PCR using the BOX-A1R primer revealed high genetic diversity of the *Pseudomonas syringae* isolates obtained from stone fruits in Montenegro. The genetic profiles did not support any grouping of the isolates, neither by their biochemical characteristics, nor by their host plant or origin. Abbasi et al. (2013) determined high genetic diversity of *Pss* isolates obtained from various stone fruit trees in Iran by rep-PCR and IS50-PCR. This study indicated that the rep-PCR technique is reliable, reproducible, and highly discriminative in assessment of the *Pss* strains diversity (Abbasi et al. 2013). Recent rep-PCR studies have also indicated that pv. *morsprunorum* strains form a more homogenous group than pv. *syringae* strains (Vicente and Roberts 2007; Gilbert et al. 2009; Kałużna et al. 2010).

Sequence analysis of the 16S rRNA gene and of housekeeping genes as well as repetitive elements have been described as useful methods for identification and classification and for diversity studies of strains belonging to *P. syringae* (Bultreys and Kałużna 2010; Hwang et al. 2005). The 16S rRNA gene was analyzed for four isolates, which did not have a gene for syringomycin synthesis. Obtained results showed these isolates belong to *Pss*.

Multilocus Sequence Analysis (MLSA) method revealed discrimination among *P. syringae* strains (Sarkar and Guttman 2004; Hwang et al. 2005; Kałużna et al. 2010). It is a recommended method for the determination of genomic relatedness among bacterial strains, where sequences of some housekeeping genes of the bacterial core genome are compared (Sarkar and Guttman 2004). MLSA of 28 representative isolates of *P. syringae*, isolated from different *Prunus* spp. and localities in Montenegro demonstrated genomic differences between the isolates that could not be related to the geographic origin or host species. Most of the isolates, with the exception of strain K6, were grouped within genomospecies 1, *P. syringae* sensu stricto, corresponding to *P. syringae* PG 2, together with the reference *Pss* strains. However, the isolates did not form a monophyletic group. They clustered in three separate clades corresponding to previously described PG 2a, 2b, and 2d (Berge et al. 2014). Isolates isolated from apricot, almond, and Japanese plum were distributed in three different clades, while peach and nectarine isolates were included into clade 2b. One isolate from Japanese plum and one from sweet cherry were grouped together with newly described species *P. cerasi* (Kałużna et al. 2016). Only strain K6, isolated from apricot, was most distinct from the others and closely related with the reference strains belonging

to PG 3, genomospecies 2, which includes stone fruit pathogens *Psm* race 1 and *P. syringae* pv. *cerasicola* as well (Ruinelli et al. 2019). However, it was positive for *syr* B gene, commonly produced by pathovar *syringae*; thus, the taxonomic status of this strain within *P. syringae* species complex should be further studied. Neither of the isolates were closely related to the quarantine peach pathogen *Psp*, nor *Psm* race 2 belonging to PG1 (Ruinelli et al. 2019). Based on Ruinelli et al. (2019) genome-based phylogenetic study, isolates obtained from *Prunus* spp. are distributed throughout PG1, PG2 and PG3 and do not form a monophyletic group within the same PG (Ruinelli et al. 2019). Hulin et al. (2018) reported that isolates of *Pss* obtained from *Prunus* were not monophyletic and were found throughout phylogroup 2. PG 2 shows the greatest host diversity, and includes most of the pathovar *syringae* strains (Hwang et al. 2005).

All tested isolates showed high level of resistance to copper, suggesting resistance development in Montenegrin *P. syringae* population. This may be due to frequent use of copper compounds in stone fruit and almond disease control programs. Copper resistance in plant pathogenic bacteria is not a rare consequence of an intensive chemical protection in fruit-cultivating regions (Sulikowska and Sobiczewski 2008; Giovanardi et al. 2015, 2017, 2018). On the contrary, bactericidal effect of streptomycin indicated that treatment based on this bactericide could potentially improve control efficacy of the disease. However, antibiotics are not permitted in plant protection in Montenegro.

Conclusions

The results of this study indicated that *Pseudomonas syringae* is present and widely spread in commercial stone fruit orchards and almond gardens in Montenegro. Wide host range and favorable climate contribute to the pathogen population survival and spread. It seemed that major stone fruit cultivars are all susceptible to this bacterium, and depending on the environmental conditions develop mild, but in some cases, severe symptoms causing death of the diseased trees. Characterization of the isolates indicated that most of the analyzed isolates belong to pv. *syringae*, although variations among them were detected in phenotypic and genetic properties. This could be a result of different population origin, sources of infection, as well as *Pss* adaptation to different hosts and climatic conditions.

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Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were primarily performed by Tamara Popović. The manuscript is part of her dissertation. The first draft of the manuscript was written by Tamara Popović and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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