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Molecular Characterization of *Pseudomonas syringae* pv. *coriandricola* and Biochemical Changes Attributable to the Pathological Response on Its Hosts Carrot, Parsley, and Parsnip

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Abstract

Bacterial leaf spot caused by the plant pathogenic bacterium *Pseudomonas syringae* pv. *coriandricola* (*Psc*) was observed on carrot, parsnip, and parsley grown on a vegetable farm in the Vojvodina Province of Serbia. Nonfluorescent bacterial colonies were isolated from diseased leaves and characterized using different molecular techniques. Repetitive element PCR fingerprinting with five oligonucleotide primers (BOX, ERIC, GTG_5, REP, and SERE) and the randomly amplified polymorphic DNA-PCR with the M13 primer revealed identical fingerprint patterns for all tested strains. Multilocus sequence analysis of four housekeeping genes (*gapA*, *gltA*, *gyrB*, and *rpoD*) showed a high degree (99.8 to 100%) of homology with sequences of *Psc* strains deposited in the Plant-Associated Microbes Database and NCBI database. The tested strains caused bacterial leaf spot symptoms on all three host plants. Host-strain specificity was not found in cross-pathogenicity tests, but the plant response (peroxidase induction and chlorophyll bleaching) was more pronounced in carrot and parsley than in parsnip.

The large vegetable family Apiaceae (the parsley family) consists of 200 genera and >2,900 plant species grown worldwide, with representative members such as carrot (*Daucus carota* L.), parsley [*Petroselinum crispum* (Mill.) Fuss], parsnip (*Pastinaca sativa* L.), celery (*Apium graveolens* L.), fennel (*Foeniculum vulgare* Mill.), and coriander (*Coriandrum sativum* L.). Bacteria causing bacterial leaf spot were previously reported on the following: (i) coriander, reported from some European countries (Cazorla et al. 2005; Nemeth et al. 1969; Taylor and Dudley 1980; Toben and Rudolph 1996), North America (Bull and Koike 2012; Cerkauskas 2009; Cooksey et al. 1991; Pernezny et al. 1997), Australia (Dennis and Wilson 1997; Gooden et al. 1995; Refshauge and Nayudu 2001; Refshauge et al. 2010), and India (Gupta et al. 2013); (ii) parsley, reported from the United States (Bull and Koike 2012; Bull et al. 2011; Koike and Bull 2011; Xu and Miller 2013) and Turkey (Bozkurt et al. 2016); (iii) celery, reported from the United States (Bull and Koike 2012; Koike and Bishop 1990; Koike et al. 1994), and Greece (Elena et al. 2008); and (iv) fennel, reported from the United States (California; Jardini et al. 2012). Symptoms of the disease consist of brown-colored, angular-shaped, small leaf spots, limited by leaf veins and visible on both sides of the leaf. Each of these diseases was considered to be caused by different bacterial pathogens, and a study of the parsley pathogen based on multilocus sequence typing (MLST) revealed that bacterial spot disease might be caused by several pathovars that have a broader host range within the Apiaceae (Bull et al. 2011). Two *Pseudomonas syringae* pathovars have thus far been reported as causal agents of bacterial leaf spot and were identified as *P. syringae* pv. *coriandricola* (*Psc*) and *P. syringae* pv. *apii* (*Psa*) (Bull et al. 2011; Elena et al. 2008; Jardini et al. 2012; Koike et al. 1994; Toben and Rudolph 1996). These pathovars differ visually from each other in their ability to produce fluorescent pigment on King's B medium; that is, *Psc* does not produce fluorescent pigment and *Psa* does (Bull et al. 2011; Gupta et al. 2013; Xu and Miller 2013). Clear separation between these two pathovars was also established using repetitive element PCR fingerprinting (rep-PCR) and MLST techniques, as previously described by Bull et al. (2011).

Some DNA profiling methods such as rep-PCR and use of randomly amplified polymorphic DNA (RAPD) have proved to be reliable techniques for the detection of genetic diversity among *P. syringae* pathovars (Gutiérrez-Barranquero et al. 2013; Louws et al. 1994). Owing to the wide distribution of these repetitive elements throughout the small genomes of prokaryotic organisms, rep-PCR is used as a fast and accurate typing technique capable of generating strain-specific fingerprints that can differentiate bacterial strains below the species or subspecies level (De Bruijn 1992; Little et al. 1998; Louws et al. 1994). Sarkar and Guttman (2004) confirmed that biochemical or physiological distinctions for reliable differentiation among *P. syringae* pathovars do not exist, and that a clearer picture can be obtained by using the MLST technique with four housekeeping genes: *gapA* (encoding glyceraldehyde-3-phosphate dehydrogenase A), *gltA* (encoding citrate synthase), *gyrB* (encoding DNA gyrase B), and *rpoD* (encoding RNA polymerase sigma factor 70).

For the first time in Serbia, a severe bacterial leaf spot disease was observed on carrot, parsnip, and parsley under wet and cool conditions in the spring of 2014, and the agent causing the disease was identified as *Psc* based on pathogenicity, biochemical tests, and sequencing data (Popović et al. 2015). Previously, only three host plant species (*C. sativum*, *Levisticum officinale*, and *Ammi majus*) had been described as susceptible to infection by *Psc* (Toben and Rudolph 1996). Our findings indicated that this pathovar has a broader host range within the family Apiaceae. In this study, *Psc* strains isolated from carrot, parsnip, and parsley in Serbia are characterized on the basis of genomic data (rep-PCR profiles and multilocus sequence analysis [MLSA]) to determine whether the populations from different hosts are homogenous and have the same origin. In addition, strain cross-pathogenicity in relation to some biochemical

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responses of the host is evaluated to test whether host–pathogen relatedness exists and to determine host susceptibility.

Materials and Methods

Bacterial strains and growth conditions. The *Psc* strains used in this study (Table 1) were isolated from three different hosts of the family Apiaceae with expressed symptoms of leaf spot disease: carrot (coded as Pc25, Pc26, and Pc29), parsnip (coded as Pc50, Pc55, and Pc57), and parsley (coded as Pc70, Pc71, and Pc72) (Popović et al. 2015). The crops from which all strains originated were grown on the same vegetable farm, in the suburban settlement of Rimski Šančevi in Vojvodina, Serbia (45.667754° N, 19.085809° E), in 2014. Strains were routinely grown in nutrient agar (NA) medium at 26°C and maintained as stock cultures stored at −20°C in Luria Bertani broth (Difco) containing sterile glycerol (20%, vol/vol).

Molecular characterization. *Genomic DNA extraction.* Genomic DNA from the tested *Psc* strains was extracted using a modified version of the hexadecyltrimethyl ammonium bromide (CTAB) procedure of Le Marrec et al. (2000) described by Dimkić et al. (2013). All bacterial isolates were grown on NA medium for 48 h at 28°C. A loopful of each pure bacterial culture was resuspended in sterile distilled water (SDW) and centrifuged at 10,000 × *g* for 5 min. The obtained pellet was resuspended in 567 µl of TE buffer (10 mM of Tris, pH 8.0, and 1 mM of EDTA) with the addition of 30 µl of 10% sodium dodecyl sulfate and 3 µl of proteinase K (20 mg/ml in 50% glycerol). The mixture was incubated at 37°C for 30 min. After the incubation period, 5 M of NaCl was added, and DNA was purified by heating at 65°C for 10 min in 300 µl of a solution of 3% CTAB and polyvinylpyrrolidone. This step was followed by chloroform extraction one time and centrifugation at 10,000 × *g* for 10 min, where two phases were formed. A further step included the addition of one-tenth volume of 3 M of Na-acetate (pH 5.0) and one volume of isopropanol, and 15 min of centrifugation at 10,000 × *g*. The final phase consisted of pellet washing with 1 ml of cold 96% ethanol and centrifugation at 10,000 × *g* for 10 min. The DNA obtained was resuspended in 50 µl of TE buffer containing 1 µl of RNase (10 mg/ml) at a concentration of 20 to 30 ng and stored at −20°C before use.

Rep-PCR. Rep-PCR with BOX, ERIC and REP (Louws et al. 1994), GTG_5 (Versalovic et al. 1994), and SERE (Rajashekara et al. 1998) primers, as well as the RAPD-PCR with primer M13 (Huey and Hall 1989), was used to determine genetic polymorphism among the tested strains originating from carrot (Pc25, Pc26, Pc29), parsnip (Pc50, Pc55, Pc57), and parsley (Pc70, Pc71, Pc72). The primers used are listed in Table 2. Amplification of DNA bands was performed in a total volume of 25 µl containing the following: 1 µl of bacterial total DNA, 2.5 µl of 10× KAPA Taq buffer, 0.5 µl of 10 mM dNTP mixture (KAPA Biosystems), 2 µl (10 µM) of primer sets (Table 1), 16.8 µl of ultrapure DNase/RNase-free water (Gibco, Fisher Scientific), and 0.2 µl (5 U/µl) of KAPA Taq polymerase (KAPA Biosystems). The PCR amplifications were performed with a Mastercycler personal model (Eppendorf) using the following conditions: an initial denaturation cycle at 95°C for 7 min (BOX, ERIC, and REP PCR), 6 min (GTG_5 PCR), or 5 min (SERE PCR) and at 94°C for 2 min (M13 PCR). This was followed by 30 cycles (BOX, ERIC, and GTG_5 PCR), 35 cycles (REP and SERE PCR), or 40 cycles (M13 PCR) of denaturation at 94°C for 1 min; primer annealing at 40°C (GTG_5 and REP PCR), 50°C (SERE PCR), and 52°C (BOX and ERIC PCR) for

Table 1. List of *Pseudomonas syringae* pv. *coriandricola* strains used in this study and NCBI and Plant-Associated Microbes Database (PAMDB) accession numbers[z]

Strain name	Host	NCBI GenBank accession numbers				PAMDB
		rpoD	*gapA*	*gltA*	*gyrB*	
Pc25	*Daucus carota* subsp. *sativus* (carrot)	–	–	–	–	–
Pc26		KM979437	MG198609	MG198612	KM979434	1807
Pc29		–	–	–	–	–
Pc50	*Pastinaca sativa* (parsnip)	–	–	–	–	–
Pc55		KM979438	MG198610	MG198613	KM979435	1808
Pc57		–	–	–	–	–
Pc70	*Petroselinum crispum* (parsley)	–	–	–	–	–
Pc71		–	–	–	–	–
Pc72		KM979439	MG198611	MG198614	KM979436	1809

[z] Dashes indicate that sequences of certain strains are not deposited into the NCBI database, and thus the accession numbers are not provided.

Table 2. Primers used in repetitive element PCR fingerprinting profiling and multilocus sequence analysis

Primer/locus name[z]	Primer sequence (5′ to 3′)	Reference
BOXAIR	CTACGGCAAGGCGACGCTGACG	Louws et al. (1994)
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	
ERIC2	AAGTAAGTGACTGGGGTGAGCG	
REP1R-I	IIIICGICGICATCIGGC	
REP2-I	ICGICTTATCIGGCCTAC	
GTG5	GTGGTGGTGGTGGTG	Versalovic et al. (1994)
SERE	GTGAGTATATTAGCATCCGCA	Rajashekara et al. (1998)
M13	GAGGGTGGCGGTTCT	Bonomo et al. (2008)
gltA (For)	AGTTGATCATCGAGGGCGCWGCC	Sarkar and Guttman (2004)
gltA (Rev)	TGATCGGTTTGATCTCGCACGG	
gapA (For)	CGCCATYCGCAACCCG	Hwang et al. (2005)
gapA (Rev)	CCCAYTCGTTGTCGTACCA	
gyrB (For)	MGGCGGYAAGTTCGATGACAAYTC	
gyrB (Rev)	TRATBKCAGTCARACCTTCRCGSGC	
rpoD (For)	AAGGCGARATCGAAATCGCCAAGCG	
rpoD (Rev)	GGAACWKGCGCAGGAGTCGGCACG	

[z] For = forward and Rev = reverse.

1 min or 42°C for 20 s (M13 PCR); and polymerization at 65°C for 8 min (ERIC, GTG_5, and REP PCR) and at 72°C for 8 min (BOX PCR) and 2 min (SERE and M13 PCR). The final extension step was performed at 65°C (ERIC, GTG_5, and REP PCR) and at 72°C (BOX and SERE PCR) for 16 min, or at 72°C for 10 min (M13 PCR). The generated fingerprints were visualized using gel electrophoresis on 1% agarose gels in 0.5× TBE buffer stained with ethidium bromide for 2 h at 90 V. For visualization, PCR products were mixed with DNA Gel Loading Dye 6× (Thermo Scientific) and visually compared.

MLSA. MLSA was performed using partial coding sequences of four housekeeping genes: *gapA*, *gyrB* and *rpoD* (Hwang et al. 2005), and *gltA* (Sarkar and Guttman 2004) (Table 2). The PCR mixture (25 μl) was composed of 12.5 μl of KAPA Taq Ready-Mix (a doubly concentrated solution of Taq DNA polymerase in 0.05 U/μl of reaction buffer with a 0.4 mM concentration of each dNTP; Kapa Biosystems), 1 μl of the primers (10 μM) in each primer set, 9.5 μl of ultrapure DNases/RNase-free water, and 1 μl of target DNA. All PCR amplifications were performed according to Sarkar and Guttman (2004) with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 2 min; annealing at 54°C (*gapA*), 56°C (*gltA*), 62°C (*gyrB*), or 63°C (*rpoD*) for 1 min; extension at 72°C for 1 min; and a subsequent final extension step at 72°C for 10 min. The obtained PCR products were checked for the presence of a band of expected size in relation to 1 kb on the GeneRuler DNA Ladder (Thermo Scientific) using electrophoresis. The products were then purified using the QIAquick PCR Purification and Gel Extraction Kits (QIAGEN GmbH, Hilden, Germany) and sent for sequencing to the Macrogen Sequencing Service in the Netherlands. The sequences generated after PCR amplification were deposited in the NCBI GenBank database and Plant-Associated Microbes Database (PAMDB) and their accession numbers are shown in Table 1.

Phylogenetic analysis. The sequences obtained from the Macrogen Sequencing Service were checked for quality, aligned using CLUSTAL W implemented in the BioEdit program (version 7.1.3), and searched for homology with sequences deposited in the PAMDB (http://genome.ppws.vt.edu/blast_MLST/blast.html) and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) databases. Using the data available in these databases, two phylogenetic trees were made to compare the tested strains with already deposited *Psc* strains and with 20 and four sequences of strains of other *P. syringae* pathovars from PAMDB and NCBI, respectively (Tables 3 and 4).

When PAMDB data were used, all of our sequences were trimmed to the following nucleotide sizes: *gapA* (476 nt), *gyrB* (507 nt), and *rpoD* (381 nt). In addition, individual alignments were performed and concatenated sequences in a total length of 1,364 nt were made and used for phylogenetic analysis. A phylogenetic tree was constructed using the neighbor-joining algorithm. The tree was constructed using Mega 7.0 software, and genetic distances were computed using the Kimura two-parameter nucleotide substitution model (Kimura 1980; Kumar et al. 2016). The tree was rooted with the sequence of an outgroup strain, *P. viridiflava* BS0001.

When NCBI data were used, our sequences were aligned and trimmed to exact sizes with those from the NCBI database (*gapA*, 476 nt; *gltA*, 310 nt; *gyrB*, 455 nt; and *rpoD*, 385 nt) and concatenated sequences were made (1,626 nt). A phylogenetic tree was constructed according to the same procedure as described above. The sequence of *P. viridiflava* strain CFBP 1590 was used as an outgroup sequence.

Pathogenicity assay. For the pathogenicity assay, plants of carrot, parsnip, and parsley were raised from seeds sown in plastic

Table 3. *P. syringae* pathovar strains from the Plant-Associated Microbes Database (PAMDB) used for multilocus sequence analysis with strains used in this study[z]

Species	Strain number	Host	Year	Country	PAMDB accession number
P. s. pv. *coriandricola*	BS456	*Petroselinum crispum* (parsley)	2003	Salinas, California (U.S.)	783
P. s. pv. *coriandricola*	BS462	*Petroselinum crispum* (parsley)	2002	Salinas, California (U.S.)	784
P. s. pv. *coriandricola*	BS821	*Petroselinum crispum* (parsley)	2005	Yuma, Arizona (U.S.)	786
P. s. pv. *coriandricola*	BS2059	–	2006	Santa Maria, California (U.S.)	787
P. s. pv. *coriandricola*	BS2091	*Petroselinum crispum* (parsley)	2006	Turlock, California (U.S.)	788
P. s. pv. *coriandricola*	BS2097	*Petroselinum crispum* (parsley)	2006	Turlock, California (U.S.)	789
P. s. pv. *coriandricola*	BS2705	*Petroselinum crispum* (parsley)	2009	King City, California (U.S.)	792
P. s. pv. *coriandricola*[pt]	BS2673 (ICMP 12471)	–	–	–	795
P. s. pv. *coriandricola*[pt]	BS2888	–	–	–	880
P. s. pv. *coriandricola*	SM69-07	*Petroselinum crispum* (parsley)	2007	Ohio (U.S.)	1138
P. s. pv. *syringae*[pt]	BS2733 (LMG 1247)	–	–	–	796
P. s. pv. *syringae*	61	*Phaseolus vulgaris* (bean)	–	–	67
P. s. pv. *syringae*	B76	*Lycopersicon esculentum* (tomato)	–	Georgia (U.S.)	73
P. s. pv. *syringae*	1212R	*Pisum sativum* (pea)	–	–	68
P. s. pv. *pisi*[pt]	BS2753 (NCPPB 2585)	–	–	–	815
P. s. pv. *pisi*	H6E5	*Pisum sativum* (pea)	1994		59
P. s. pv. *apii*[pt]	BS2889 (NCPPB 1626)	*Apium graveolens* var. *dulce* (celery)	1942	U.S.	908
P. s. pv. *aptata*	601	*Beta vulgaris* (sugarbeet)	1966	–	94
P. s. pv. *aptata*[pt]	BS291 (CFBP 1617)	–	–	–	776
P. s. pv. *atrofaciens*	DSM5025	*Triticum aestivum* (wheat)	–	–	3
P. s. pv. *atrofaciens*[pt]	BS2457 (LMG 5095)	–	–	–	794
P. s. pv. *glycinea*[pt]	BS2748 (LMG 5066)	–	–	–	810
P. s. pv. *glycinea*	LN10	*Glycine max* (soybean)	1994		15
P. s. pv. *phaseolicola*[pt]	BS2739 (LMG 2245)	–	–	–	801
P. s. pv. *phaseolicola*	HB10Y	*Phaseolus vulgaris* (bean)	–	–	49
P. s. pv. *lachrymans*	107	*Cucumis sativus* (cucumber)	–	–	21
P. s. pv. *tabaci*[pt]	BS2917 (NCPPB 1427)	–	–	–	890
P. s. pv. *tabaci*	LMG5393	*Nicotiana tabacum* (tobacco)	1959	Hungary	879
P. s. pv. *tomato*[pt]	BS287 (CFBP 2212)	–	–	–	882
P. s. pv. *helianthi*[pt]	LMG 5067	–	–	–	811
P. viridiflava	BS0001	*Actinidia deliciosa* (kiwifruit)	2008	Brescia, Lombardy (Italy)	1140

[z] The superscript pt indicates pathotype strain for certain species. Dashes indicate that information for a given item does not exist.

pots filled with a fertilized substrate (Domoflor Mix 6; Domoflor UAB, Vilnius, Lithuania). Pots were placed in a growth chamber under controlled conditions of 30°C/20°C (day/night) temperature, a 16-h daylight regime, light intensity of 2,700 to 3,000 lx, and 30 to 40% RH. Plants were inoculated 5 weeks after sowing, in the three to five true leaf stage (BBCH 13-15), using a method involving spraying inocula onto leaf surfaces until runoff occurred. Bacterial inocula were prepared from strains grown on nutrient sucrose agar (NSA) at 27°C for 24 h and suspended in 250 ml of SDW to a concentration of approximately 10^8 CFU/ml. The plants were kept in a highly humid condition (100% RH) for 6 h before and 48 h after the inoculation. After that, they were kept at a temperature of 22 ± 2°C and RH of 70 to 80%. Five plants of each host were tested for each of the nine representative *Psc* isolates. The experiment was performed in four replications. Plants treated with SDW served as a negative control. The plants were observed daily for symptom formation over a period of 3 weeks. Pathogenicity was checked in two independent experiments (each in four replicates).

Plants were checked visually for the presence of bacterial spot symptoms 21 days after inoculation using the Horsfall-Barratt rating scale (Horsfall and Barratt 1945), which was originally designed to compensate for error in interpretation of the percentage of infected foliage and converted to a percentage using so-called Elanco tables (Redman et al. 1969) (Table 5). Numerical values were given for the percentage of leaf area showing disease symptoms and then converted using midpoint percentages prior to statistical analyses.

The analysis of variance was supported by the Kolmogorov-Smirnov test for the normality of residuals. The obtained data were subjected to one-way analysis of variance, and separation of means of the percentage of infection in planta was accomplished using Tukey's honest significant difference test. Results were considered significant at $P < 0.05$. Statistical analyses were conducted using the general procedures of STATISTICA version 7 (StatSoft Inc.) and IBM SPSS Statistics version 20 (SPSS Inc.).

Reisolations were performed from leaves of each host showing bacterial leaf spot symptoms. One bacterial reisolate from each inoculated host was taken to confirm its being identical to the original isolates using so-called LOPAT biochemical tests (Lelliott and Stead 1987; Schaad et al. 2001) and M13 PCR fingerprinting.

Biochemical changes in inoculated hosts. *Peroxidase extraction and electrophoresis.* Symptomatic and asymptomatic fresh leaves of carrot, parsley, and parsnip previously inoculated with the tested strains (Pc26, Pc55, Pc72) or SDW (negative control) were frozen in liquid nitrogen, powdered in a mortar, and extracted in 100 mM of sodium phosphate buffer (pH 6.5) with 2 mM of phenylmethylsulphonyl fluoride. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was used for measurement of class III peroxidase (POD) (E.C.1.11.1.7) activity and separation of POD isoforms. POD activity of the soluble protein fraction was measured spectrophotometrically with guaiacol as a substrate at 25°C using a Shimadzu UV-160 temperature-controlled spectrophotometer (Shimadzu, Kyoto, Japan). The reaction mixture consisted of 100 mM of Na-phosphate buffer (pH 6.5), 0.01 M of guaiacol, and a 20-µl aliquot of a sample. The reaction was started by adding H_2O_2 to 10 mM, and the increase in absorbance at 470 nm was then followed. POD activity was calculated using the extinction coefficient for guaiacol oxidation products (26.6 mM^{-1}) (Kukavica and Jovanović 2004).

Isoelectrofocusing of soluble POD from leaf extracts was carried out in a 7.5% (wt/vol) polyacrylamide gel with 3 M of urea and 2% ampholyte (Serva, Heidelberg, Germany) at 4°C for 3 h. Isoelectrofocusing was performed at a gradually increasing voltage: 90 min

Table 5. Horsfall-Barratt scale used to estimate the severity of bacterial leaf spot disease[z]

Rating	Infected leaf area (%)	Range	Midpoint (Elanco formula)
0	0	0	0
1	>0 to 3	3	1.5
2	>3 to 6	3	4.5
3	>6 to 12	6	9.0
4	>12 to 25	13	18.5
5	>25 to 50	25	37.5
6	>50 to 75	25	62.5
7	>75 to 87	12	81.5
8	>87 to 94	7	91
9	>94 to 97	3	96.5
10	>97 to 100	3	98.5
11	100	0	100

[z] See Horsfall and Barratt (1945) and Redman et al. (1969).

Table 4. *P. syringae* pathovar strains from the NCBI database used for multilocus sequence analysis with strains used in this study

Species	Strain number	Host	Country	NCBI database accession number			
				gapA	*gltA*	*gyrB*	*rpoD*
P. s. pv. *coriandricola*	SUPP3309	*Coriandrum sativum* (coriander)	Shizuoka (Japan)	LC312432	LC321982	LC310752	LC312425
P. s. pv. *coriandricola*	SUPP3326	*C. sativum* (coriander)	Shizuoka (Japan)	LC312428	LC321978	LC311058	LC312421
P. s. pv. *coriandricola*	SUPP3327	*C. sativum* (coriander)	Shizuoka (Japan)	LC312429	LC321979	LC311059	LC312422
P. s. pv. *coriandricola*	SUPP3328	*C. sativum* (coriander)	Shizuoka (Japan)	LC312430	LC321981	LC311060	LC312423
P. s. pv. *coriandricola*	SUPP3330	*C. sativum* (coriander)	Shizuoka (Japan)	LC312431	LC321980	LC311061	LC312424
P. s. pv. *coriandricola*	SUPP3344	*C. sativum* (coriander)	Chiba (Japan)	LC312433	LC321983	LC311062	LC312426
P. s. pv. *coriandricola*	SUPP3345	*C. sativum* (coriander)	Chiba (Japan)	LC312434	LC321984	LC311063	LC312427
P. s. pv. *tomato*	CFBP2212^T	*Lycopersicon esculentum* (tomato)	U.K.	KF937402	KF937499	KF937596	KF937693
P. s. pv. *maculicola*	5309-1	*Brassica napus* (oilseed rape)	Canada	KJ641615	KJ634662	KJ634656	KJ634659
P. s. pv. *syringae*	LMG 1247^T	*Syringa vulgaris* (lilac)	U.K.	HG000210	HG000145	HG000275	HG000080
P. s. pv. *syringae*	B728a	*Phaseolus vulgaris* (snap bean)	Wisconsin (U.S.)	NC_007005	NC_007005	NC_007005	NC_007005
P. s. pv. *syringae*	LMG 1247	*S. vulgaris* (lilac)	U.K.	HG000210	HG000145	HG000275	HG000080
P. s. pv. *pisi*	ATCC 11043	*Pisum sativum* (peas)	U.S.	KP211403	KP211407	KP211405	KP211409
P. s. pv. *atrofaciens*	LMG5095	*Triticum aestivum* (wheat)	New Zealand	NZ_CP028490	NZ_CP028490	NZ_CP028490	NZ_CP028490
P. viridiflava	CFBP 1590	*Prunus cerasus* (cherry)	France	LT855380	LT855380	LT855380	LT855380

at 100 V, 60 min at 250 V, and 30 min at 500 V. The anode buffer was 10 mM of phosphoric acid and the cathode buffer was 20 mM of NaOH with 3% ampholyte on a pH gradient of 3 to 9 (Kukavica et al. 2012). To detect POD activity after electrophoresis, the gel was incubated with 10% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM of sodium phosphate buffer (pH 6.5) (Kukavica et al. 2012).

Chlorophyll and flavonoid measurements. Total chlorophyll content and content of leaf epidermal flavonoids (EpFlav, DA_{375}) were measured in vivo with a Dualex FLAV(FORCE-A) apparatus (Orsay, France) (Cerović et al. 2012). Measurements were taken from 7 to 10 leaves per repetition on days 3, 12, and 20 after inoculation (Cerović et al. 2012).

Results

Molecular characterization. The results of rep- and RAPD-PCR fingerprinting analysis for all six primer pairs used (BOX,

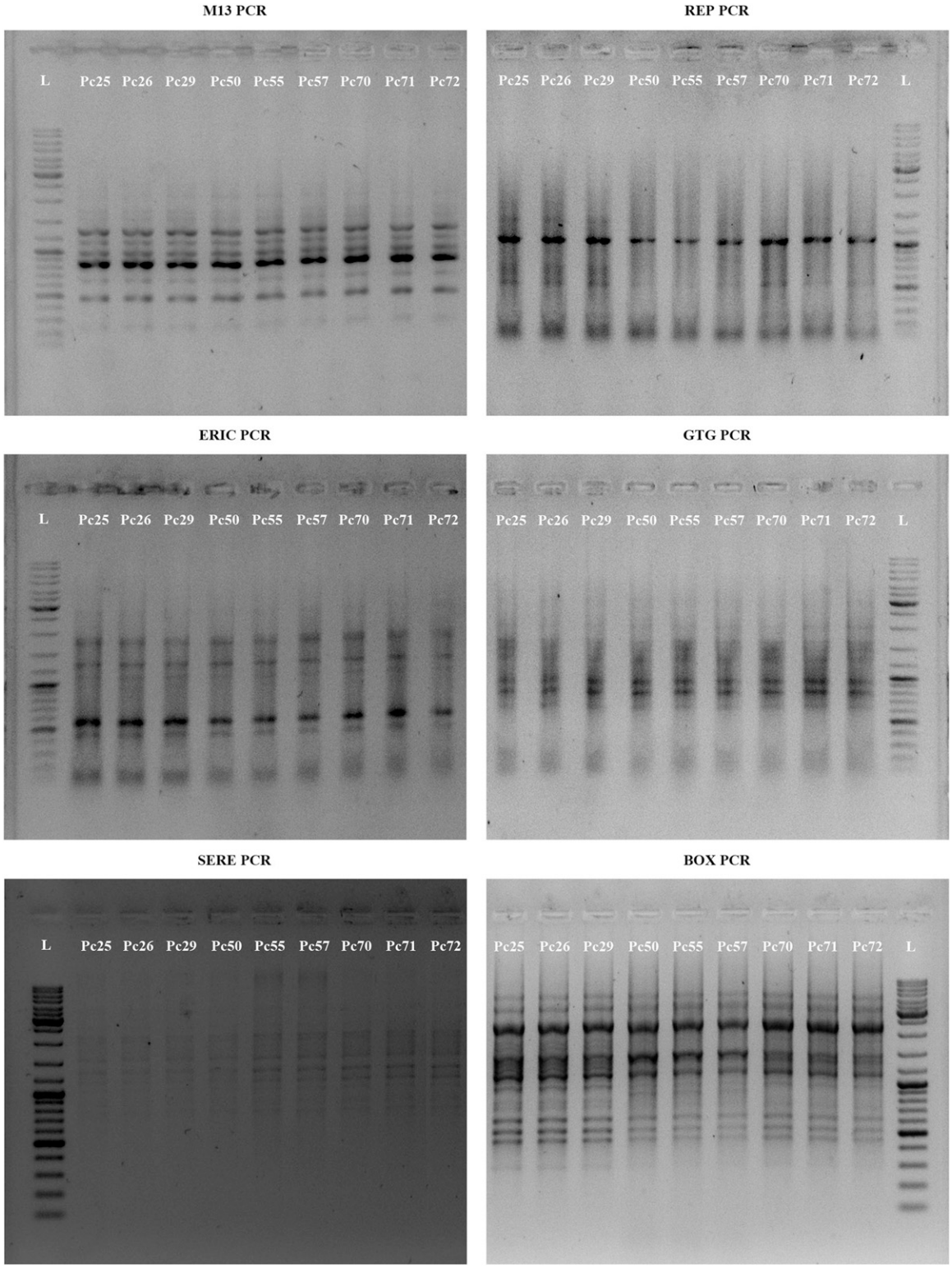

Fig. 1. Results of randomly amplified polymorphic DNA and repetitive element PCR fingerprinting using six primers for each of the tested *Pseudomonas syringae* pv. *coriandricola* strains, originating from carrot (Pc25, Pc26, Pc29), parsnip (Pc50, Pc55, Pc57), or parsley (Pc70, Pc71, Pc72). L = 1-kb GeneRuler DNA ladder.

ERIC, GTG_5, REP, SERE, M13) are shown in Figure 1. The DNA profiles were visually identical using all of the tested primers, revealing high genetic homogeneity between the tested *Psc* strains isolated from carrot, parsnip, and parsley in Serbia. It turned out that BOX, SERE, and M13 PCR fingerprinting techniques generated the most complex banding patterns, with an average of 12, 8, and 10 bands, respectively, and with amplification size between 400 and 4,000 bp.

In PAMDB, BLAST analysis of the *gapA* and *gyrB* sequences showed 100% homology among all *Psc* strains (BS456, BS426, BS821, BS2059, BS2091, BS2097, 2075, ICMP 12471, BS2888, and SM69-07), whereas the *rpoD* gene showed 99% homology with the sequence of *Psc* pathotype strains ICMP 12471 and BS2888, as well as with that of strain SM69-07. Sequencing of the *gltA* gene determined the strains only to the species level (*P. syringae*) with 96% homology, indicating higher discriminatory

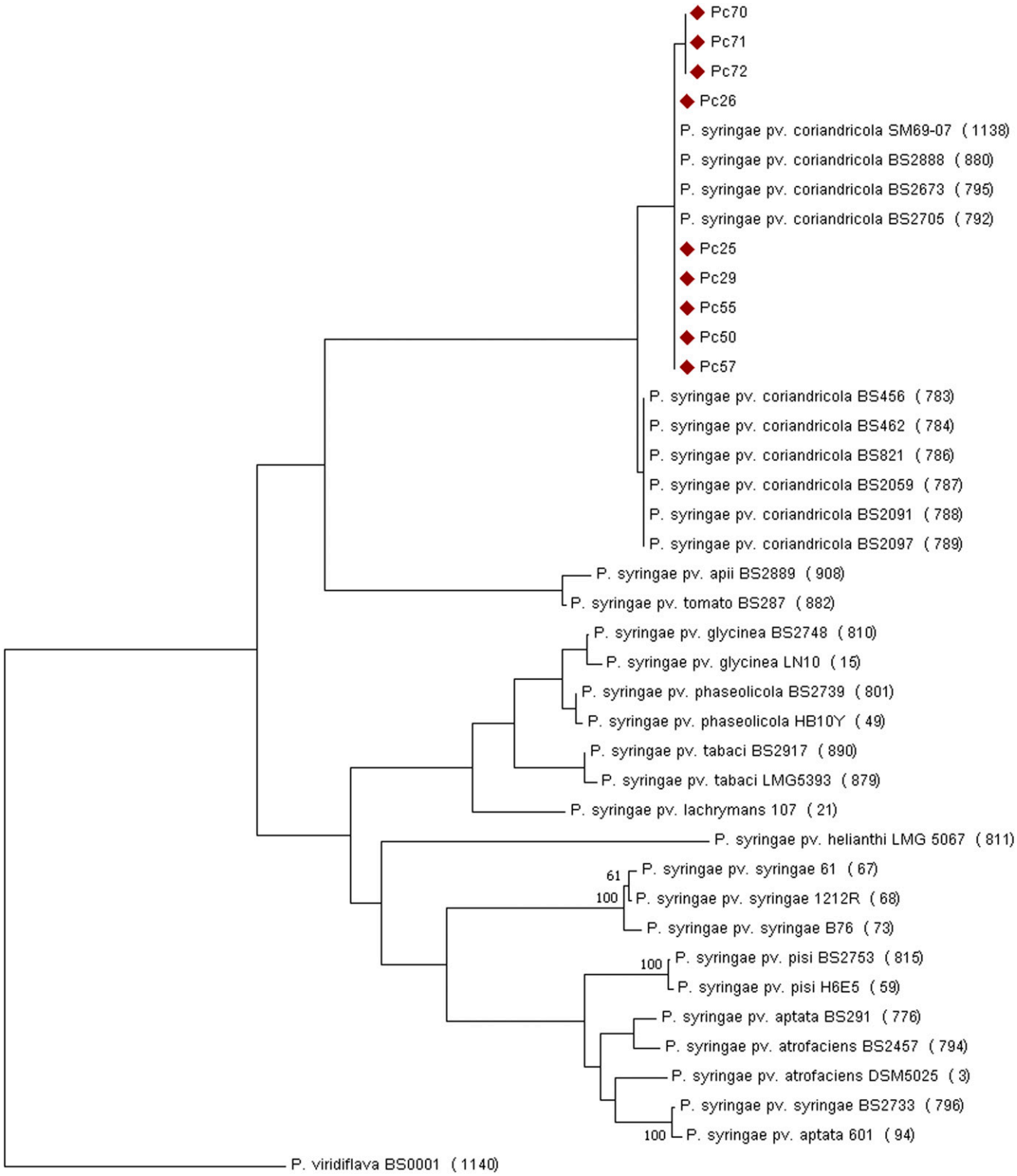

Fig. 2. Neighbor-joining phylogenetic tree based on the concatenated sequences of *gapA*, *gyrB*, and *rpoD* from tested *Pseudomonas syringae* pv. *coriandricola* strains and selected *P. syringae* pathovars deposited in the Plant-Associated Microbes Database. The tree was rooted with *P. viridiflava* BS0001 as an outgroup strain.

ability of the *gapA*, *gyrB*, and *rpoD* genes. A neighbor-joining phylogenetic tree constructed with concatenated sequences of the tested *Psc* strains and those of strains from PAMDB (Table 3) showed two closely related phylogenetic groups of *Psc*, one with all nine tested strains originating from carrot, parsley, and parsnip, two pathotype strains (BS2673 and BS2888), and two additional strains (SM69-07 and BS2705) from parsley (Fig. 2). The second group consisted of the remaining *Psc* strains from curled-leaf (BS456), flat-leaf (BS462, BS821, BS2097), and double-curled (BS2091) parsley and strain BS2059 (origin not defined) used for comparison. Two *P. syringae* pathovars, *apii* (BS2889) and *tomato* (BS287), were phylogenetically more closely related to *Psc* strains than the other nine *P. syringae* pathovars (*aptata*, *atrofaciens*, *glycinea*, *helianthi*, *lachrymans*, *pisi*, *phaseolicola*, *syringae*, and *tabaci*), which were grouped in the second phylogenetic tree cluster.

In the NCBI database, the tested *Psc* strains from Serbia on all four loci (*gapA*, *gltA*, *gyrB*, and *rpoD*) showed 100% homology with *Psc* strains SUPP3309, SUPP3326-3328, SUPP3330, SUPP3344, and SUPP3345. A concatenated neighbor-joining phylogenetic tree with the tested *Psc* strains and strains from other pathovars (*atrofaciens*, *maculicola*, *syringae*, *pisi*, and *tomato*) retrieved from the NCBI database (Table 4) grouped all *Psc* strains together (Fig. 3). Other *P. syringae* pathovars were grouped in separate tree clusters, one with *P. syringae* pv. *tomato* (CFBP2212) and *maculicola* (5309-1) type strains, which were phylogenetically more closely related to *Psc* strains, and the second cluster with two *Psc* strains (B728a and LMG 1247^{T}) and pathovars *atrofaciens* (LMG5095) and *pisi* (ATCC 11043), showing their relatedness. The outgroup strain *P. viridiflava* (CFBP 1590) was situated on an isolated tree branch.

Pathogenicity assay. All nine of the tested strains of *Psc* caused symptoms in the guise of leaf spots on all three host plant species. Symptoms started appearing 8 days after inoculation as small water-soaked spots on the inoculated leaves. Leaf symptoms similar to those on the original plants were observed for 21 days after inoculation, during which the plants were rated. Disease intensity differed, not between strains, but rather between hosts (Fig. 4). Parsley and carrot were more susceptible, with disease intensity ranging from 24.3 to 34.5% and from 22.9 to 32.6%, respectively (Figs. 4 and 5), whereas that of parsnip was from 1.7 to 2.9%. Symptoms did not develop on the negative control plants treated with SDW.

Nine nonfluorescing bacterial reisolates obtained from each of the inoculated hosts were confirmed to be identical to the original isolates using the LOPAT tests, where reactions corresponded to LOPAT group Ia (+—+). Results of M13 PCR fingerprinting revealed the same banding patterns as the original ones. Target bacteria were not isolated from the negative control plants.

Biochemical changes in inoculated hosts. Chlorophyll content decreased by about 35% in carrot plants inoculated with strains Pc26 (carrot) and Pc72 (parsley), whereas chlorosis was not developed when plants were inoculated with strain Pc55 (parsnip) (Table 6). On the other hand, in diseased parsley and parsnip plants, chlorophyll content did not change significantly compared with the negative control plants throughout the whole experiment.

Induction of phenolic biosynthesis is another indicator of abiotic and biotic stresses in plants. We measured the accumulation of leaf epidermal flavonoids with the aim of using it as an indicator of the activation of phenolic metabolism. Compared with parsnip, leaves

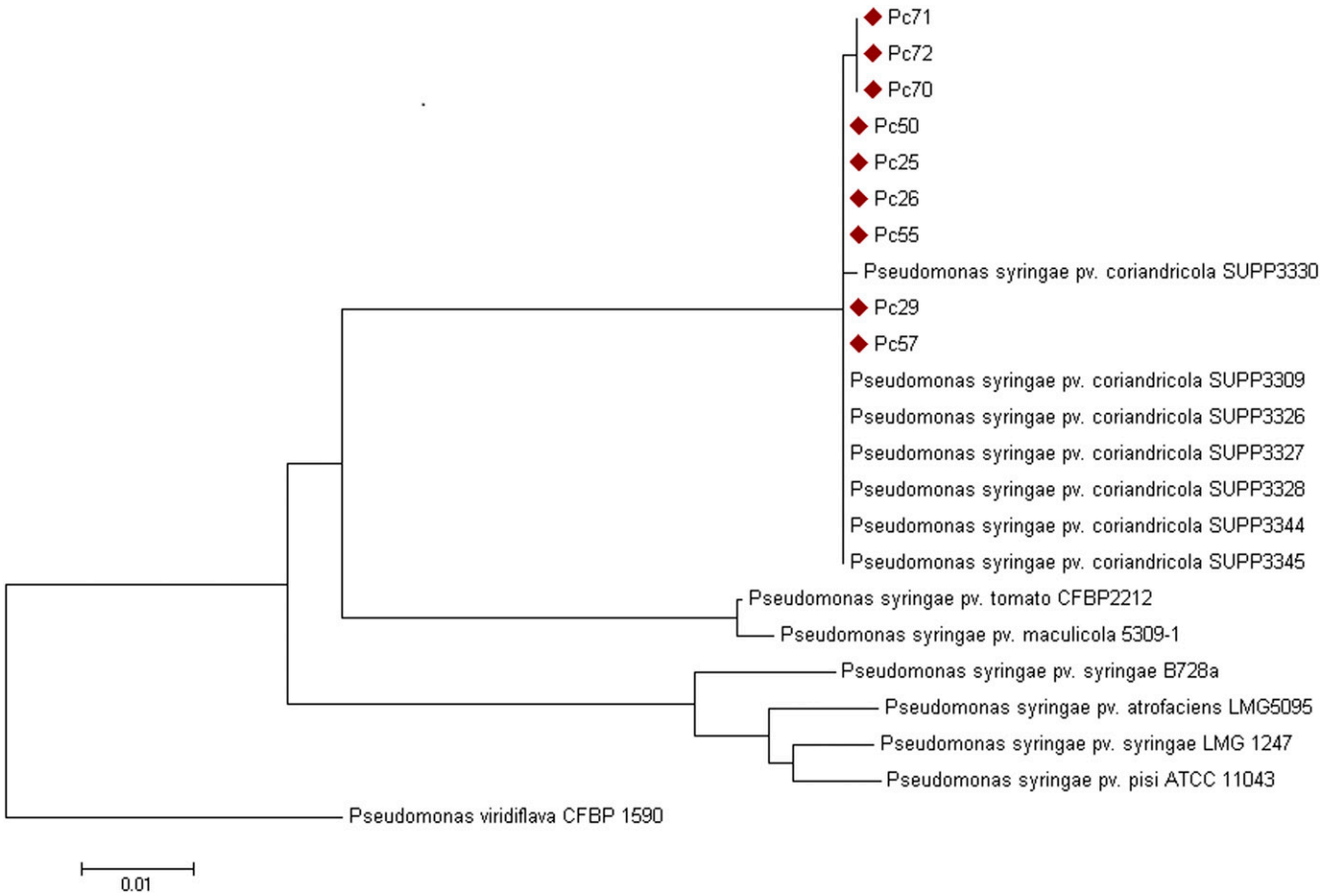

Fig. 3. Neighbor-joining phylogenetic tree based on the concatenated sequences of *gapA*, *gltA*, *gyrB*, and *rpoD* from tested *Pseudomonas syringae* pv. *coriandricola* strains and the closest *P. syringae* pathovars deposited in the NCBI database. The tree was rooted with *P. viridiflava* CFBP 1590 as an outgroup strain.

of carrot and parsley accumulated greater amounts of epidermal flavonoids, the levels of which decreased by 50% on days 3 and 12 in diseased carrot and parsley, respectively. Parsley leaves recovered flavonoids in the epidermis 3 weeks after inoculation, but only in those plants inoculated with Pc26 (carrot) and Pc72 (parsley). Similar changes were observed in parsnip. The level of total class III POD activity in symptomatic and asymptomatic carrot leaves infected with *Psc* strain Pc26 isolated from carrot, measured 20 days after inoculation, increased by 30%. In the other two plant species (parsley and parsnip), POD activity did not change significantly in asymptomatic leaves, whereas it doubled in parsnip and parsley leaves where visible symptom (spots) were present (Fig. 6). In all cases, no new inducible isoforms were found (*data not shown*).

Discussion

P. syringae is a phytopathogenic bacterium responsible for a variety of bacterial spot, speck, and blight symptoms with a very large host range (Sarkar and Guttman 2004). Because of the heterogeneous nature of this species, it is hard to determine *P. syringae* pathovars relying only on classical methods of determination based on phenotypic and biochemical characterization. This study is an upgrade and continuation of earlier research that complements the first report of *Psc* on carrot, parsley, and parsnip in Serbia, where this pathovar was identified based on colony morphology, biochemical tests, and pathogenicity (Popović et al. 2015). For better insight into genetic features of bacterial species, many molecular techniques, such as DNA-DNA hybridization and MLSA of housekeeping genes (Bull et al. 2011; Chapman et al. 2012; Gardan et al. 1999), have been proposed. Highly conservative DNA elements BOX, ERIC, REP, GTG_5, and SERE are ubiquitous and widely distributed through the genome of many plant pathogenic bacteria and therefore can be used as reliable tools for rapid and simple fingerprint comparison, especially where differentiation based on phenotypic features does not give enough information (De Bruijn 1992; Louws et al. 1994). All six PCR fingerprinting primers used in this study showed identical DNA banding patterns, demonstrating high genetic homogeneity among the tested *Psc* strains regardless of host affiliation. It turned out that BOX, SERE, and M13 PCR generated the most complex and visually most receptive patterns. Thus, BOX, SERE, or M13 PCR primers are the best primers for fast screening of *Psc* strains and can be recommended for future genetic analysis of *Psc*. The BOX and ERIC oligonucleotide primers generated DNA fingerprinting patterns identical to those obtained in a study of typing of *Psc* strains isolated from parsley (Bull et al. 2011). Significant results in separation of *P. syringae* into genomospecies were previously confirmed using the BOXA1R rep-PCR fingerprinting primer (Marques et al. 2000). Division of *P. syringae* species into nine genomospecies was also confirmed based on DNA-DNA hybridization (Gardan et al. 1999).

In many earlier studies, rep-PCR was successfully used together with MLSA for typing of different *P. syringae* pathovars (Bull et al. 2011; Marques et al. 2008; Morris et al. 2000). In our study, the four housekeeping genes *rpoD*, *gapA*, *gltA*, and *gyrB* proved to be reliable tools for pathovar identification, since all tested strains

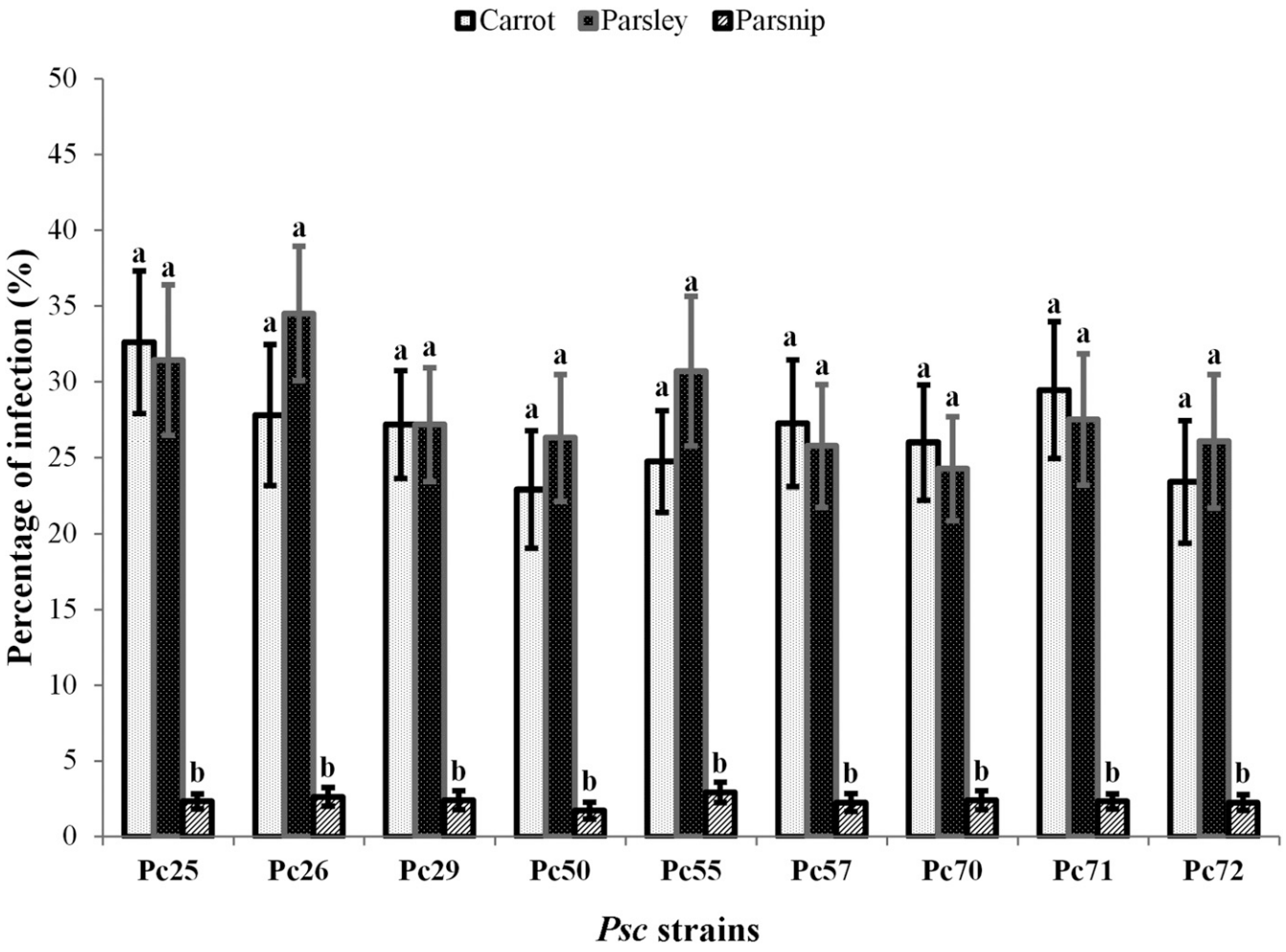

Fig. 4. Disease intensity (mean ± SE) on parsley, carrot, and parsnip 21 days after inoculations with *Pseudomonas syringae* pv. *coriandricola* bacterial strains. Values followed by the same letter in each group of columns and isolates are not significantly different ($P < 0.05$), according to Tukey's honest significant difference test.

were identified as *Psc* with similarity from 99.81 to 100% (query coverage, 98%; expectation value, 0.0) compared with data deposited in NCBI and 100% (*rpoD*, *gapA*, *gyrB*) when compared with the PAMDB database. The PAMDB BLAST results managed to identify the *gltA* gene sequences obtained in the present study only to the species level (*P. syringae* [100%]) because all of the existing *Psc* strains previously deposited in PAMDB were amplified using a different *gltA* primer (Morris et al. 2000), and therefore no data are available for comparison with our sequences, which were amplified using the *gltA* primer proposed by Hwang et al. (2005). Although PAMDB was specially designed for MLST and MLSA of plant pathogens, it contains no data on *gltA* gene sequencing and is unable to identify tested bacteria on the pathovar level, contrary to the NCBI database, where BLAST results revealed 100% identity (query coverage, 98%; expectation value, 0.0) with the deposited *Psc gltA* sequences. Based on all of the aforementioned facts, it can be asserted that the existence of a database like PAMDB, specially created for plant pathogenic bacteria, containing not only MLST results but also morphology and pathogenicity data as well, could speed up the search for needed results. It is easier to find data required for MLSA in this database than in NCBI, but it is necessary to constantly enrich it with new data to improve its quality. Bull et al. (2011) tested the ability of MLST to identify *Psc* causing bacterial spot disease on parsley and emphasized that MLST and phylogenetic analysis based on the concatenate sequences of four housekeeping genes (*gap1*, *gltA*, *gyrB*, and *rpoD*) is a powerful diagnostic tool for allocation of *P. syringae* pathovars to a certain genomospecies, also indicating the possibility that *Psc* could belong to genomospecies 9, together with *P. cannabina* and *P. cannabina* pv. *alisalensis*. This means that *Psc* could be a novel pathovar within the species *P. cannabina*, but also stresses the need for further host-range testing before a final conclusion can be reached.

Fig. 5. Leaf spot symptom on inoculated plants of **A,** parsley, **B,** carrot, and **C,** parsnip 21 days after artificial inoculation, using a 10^8 cells/ml suspension.

Previous studies reported that *Psc* causes bacterial leaf spot diseases in members of the family Apiaceae such as parsley, celery, and coriander or cilantro (Bull et al. 2011; Toben and Rudolph 1996). Apart from parsley, carrot and parsnip were also reported in a 2015 study (Popović et al. 2015). In our present study, cross-pathogenicity testing using strains that originated from parsley, carrot, and parsnip did not demonstrate host specificity. After host range testing, Bull et al. (2011) found that *Psc* strains from parsley and the *Psc* pathotype strain ICMP 12471 (= CFBP 5010 = NCPPB 3781) isolated in 1965 from *C. sativum* var. *microcarpur* in Germany were also pathogenic on celery (*Apium graveolens* L.) and coriander or cilantro (*C. sativum* L.), causing similar leaf spot diseases. Cilantro strains of *Psc* caused disease on parsley, celery, and cilantro (Koike and Bull 2011). According to Bull et al. (2011), the expanded host range of the pathogen and the potential for reciprocal infections on the original hosts from which they were isolated have epidemiological and practical implications, for example, in the intensive and extensive vegetable industry, where multiple plantings of parsley, celery, or cilantro adjacent to or in rotation with each other can be made throughout a long growing season. Diseased plantings of any of these crops therefore may have significant impacts on adjacent and subsequent plantings. Thus, host-range information is important to farmers and field advisors, who need to take steps to prevent disease and maximize commodity quality (Bull et al. 2011). Some studies reported contrary observations to the effect that *Psc* strains from coriander (cilantro) produced minor or no symptoms on celery (Cerkauskas 2009; Toben and Rudolph 1996). Moreover, host-range studies conducted with coriander *Psc* strains on celery, carrot, fennel, parsley, and parsnip indicated that symptoms did not develop on any of these hosts (Gupta et al. 2013).

Because the pathogen is also seedborne (Bull et al. 2011; Lacy et al. 1996; Taylor and Dudley 1980), it may have been introduced in Serbia by contaminated seeds. In view of the fact that *Psc* is not restricted to the original host (coriander), which is not grown in Serbia (available official data), we can suppose that the source of infections with *Psc* is parsley, since there are a few reported records of the disease on it (Bull and Koike 2012; Bull et al. 2011; Koike and Bull 2011; Xu and Miller 2013). It is known that *Psc* can cross-infect *Apiaceae* crops under favorable environmental conditions, and it could be possible that the pathogen was splashed onto adjacent carrot and parsnip crops.

The effects of *Psc* isolates on chlorophyll content, epidermal flavonoids, and total POD activity presented in this work varied depending on the examined host species and *Psc* strains, which

suggests a differential plant response to the pathogen. One of the typical disease symptoms, leaf chlorosis (Katagiri et al. 2002), was obtained only in carrot inoculated with isolates that originated from carrot (Pc26) and parsley (Pc72).

As enzymes adapted to a wide spectrum of phenolic substrates, PODs are capable of oxidizing hydroxycinnamic derivates, flavonoids, and other phenolic compounds with different specificities (Bernards et al. 1999). Induction of POD activity during incompatible plant–pathogen interactions is well documented and often correlates with the appearance of new isoforms (Bestwick et al. 1998; Kawano 2003). The increased POD content present in leaves of all examined plant species inoculated with *Psc* was correlated with accumulation of epidermal flavonoids in leaves inoculated with Pc26 (carrot) and Pc72 (parsley). Phenolic compounds are oxidized to brown compounds (polymerized phenolics) or to more reactive species, quinones, by phenol oxidases and PODs (Takahama and Oniki 2000). The transient decreased epidermal flavonoid content obtained on day 12 after inoculation may be a result of such reactions in the epidermal layer and apoplast. However, we obtained an increase of POD activity by 100% without induction of new isoforms in symptomatic leaves. Taken together, biochemical analysis of the response to *Psc* of the three examined species implies that different mechanisms are involved in their interaction with the given plant pathogenic bacteria. The response mechanism involving specific POD reactions in the apoplast and cell wall as the first barrier to *Psc* in leaves of carrot, parsley, and parsnip will be further analyzed in planned future work.

Table 6. Effect of disease caused by *Pseudomonas syringae* pv. *coriandricola* strains on chlorophyll content and epidermal flavonoids in carrot, parsley, and parsnip[z]

Inoculated host and used strains	Day 3		Day 12		Day 20	
	Chlorophyll	Epidermal flavonoids	Chlorophyll	Epidermal flavonoids	Chlorophyll	Epidermal flavonoids
Carrot						
Negative control	7.37 ± 2.52	0.13 ± 0.04	11.50 ± 7.54	0.41 ± 0.09	11.76 ± 1.93	0.30 ± 0.05
Pc55	6.95 ± 1.31	0.16 ± 0.03	11.37 ± 0.56	0.23 ± 0.02	10.13 ± 1.48	0.32 ± 0.06
Pc72	4.83 ± 1.34	0.19 ± 0.02	8.93 ± 1.80	0.19 ± 0.02	9.40 ± 1.41	0.30 ± 0.04
Pc26	4.13 ± 1.88	0.13 ± 0.01	5.52 ± 0.86	0.18 ± 0.03	9.80 ± 3.52	0.36 ± 0.07
Parsley						
Negative control	10.22 ± 1.58	0.51 ± 0.07	11.77 ± 1.36	0.41 ± 0.09	16.53 ± 5.27	0.40 ± 0.04
Pc55	8.86 ± 2.18	0.18 ± 0.02	10.75 ± 1.71	0.24 ± 0.02	14.68 ± 2.09	0.47 ± 0.07
Pc72	7.52 ± 0.86	0.16 ± 0.02	14. 30 ± 2.82	0.19 ± 0.02	15.07 ± 2.91	0.77 ± 0.06
Pc26	11.47 ± 1.20	0.18 ± 0.02	11.28 ± 1.38	0.18 ± 0.03	17.02 ± 3.63	0.58 ± 0.08
Parsnip						
Negative control	8.73 ± 0.67	0.04 ± 0.006	11.24 ± 0.74	0.11 ± 0.04	11.66 ± 1.24	0.18 ± 0.04
Pc55	7.25 ± 1.12	0.03 ± 0.008	9.76 ± 0.86	0.08 ± 0.01	12.04 ± 1.10	0.18 ± 0.03
Pc72	9.61 ± 0.73	0.04 ± 0.007	8.98 ± 1.29	0.08 ± 0.02	11.80 ± 1.32	0.47 ± 0.03
Pc26	9.87 ± 0.88	0.05 ± 0.008	9.28 ± 0.87	0.18 ± 0.04	12.43 ± 0.74	0.26 ± 0.04

[z] Values represent means ±SE.

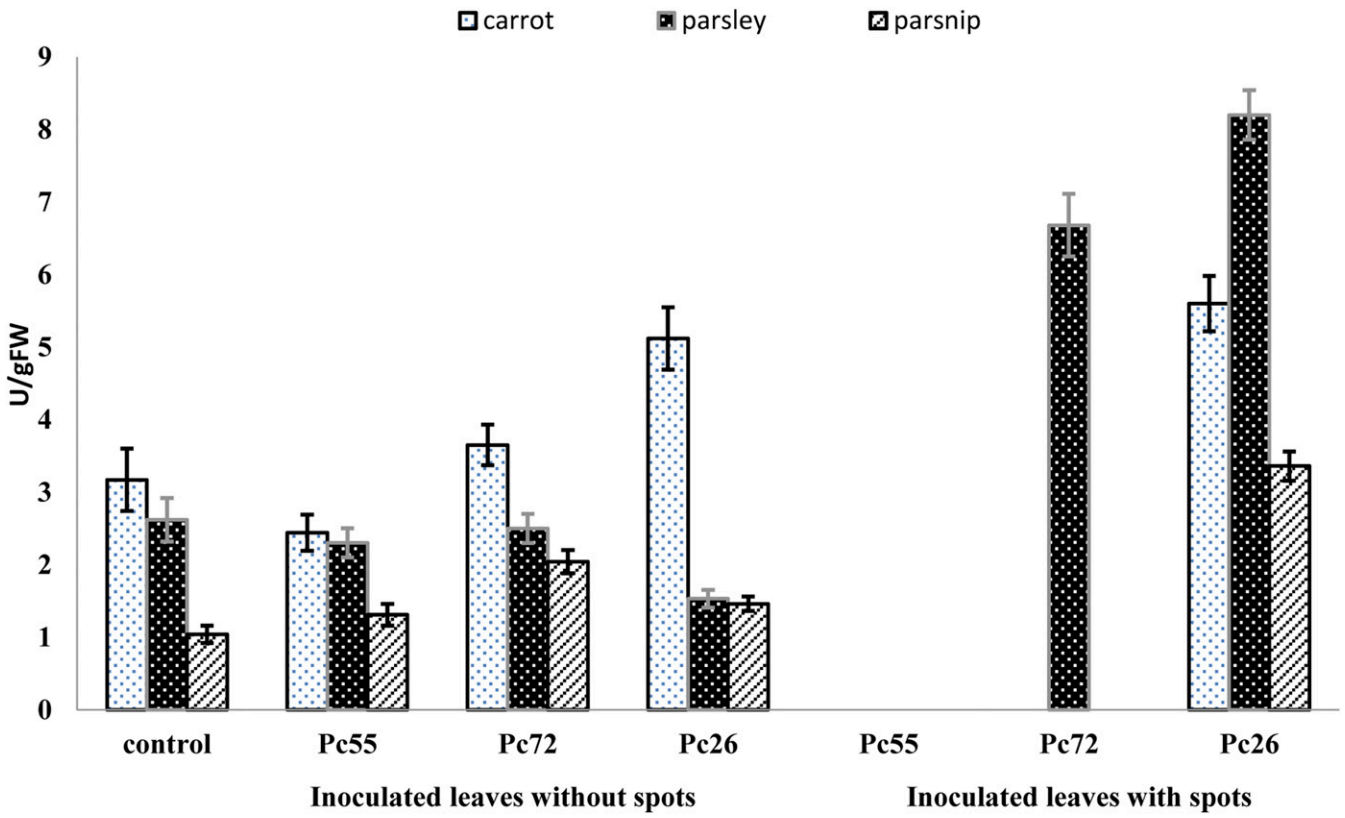

Fig. 6. Changes in the peroxidase activity in leaves of parsley, carrot, and parsnip measured on day 20 after inoculation with strains of *Pseudomonas syringae* pv. *coriandricola*.

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