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Scientific paper

Differentiation of *Pseudomonas syringae* Pathovars Originating from Stone Fruits

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SUMMARY

Due to an overlapping host range, similar symptomatology and many common characteristics, *Pseudomonas syringae* pathovars originating from stone fruits can easily be misidentified. In order to select tests for rapid and efficient differentiation of *P. s.* pvs. *syringae*, *morsprunorum* and *persicae*, we studied the suitability and differentiating potential of some standard bacteriological and molecular methods. Differentiation of the strains was performed using LOPAT, GATTa and ice nucleation tests, nutrient sucrose broth growth and utilization of various carbon sources. PCR method enabled the detection of toxin-producing genes: *syrB* and *syrD* in *P. s.* pv. *syringae*, and *cfl* gene in *P. s.* pv. *morsprunorum* race 1. Syringomycin production by pv. *syringae* was confirmed in bioassay using *Geotrichum candidum*, *Saccharomyces cerevisiae* and *Rhodotorula pilimanae* as indicator organisms. Pathogenicity test on lemon and immature nectarine fruits, as well as on string bean pods, showed different intensity of reaction of the inoculated material which could separate pv. *syringae* from the other two pathovars. PCR-based repetitive sequences, Rep-PCR with REP, ERIC and BOX primers revealed different genetic profiles within *P. syringae* pathovars.

Keywords: *Pseudomonas syringae*; Stone fruit; Identification; PCR

INTRODUCTION

Pseudomonas syringae is a polyphagous phytopathogenic bacterium associated with more than 180 species of both annual and perennial crops, including vegetables, fruits and ornamental plants (Agrios, 2005). High heterogeneity of *P. syringae* strains has resulted in species division into 57 pathovars (Gardan et al., 1997) and nine genomospecies (Gardan et al., 1999). The bacterium causes economically important diseases of cultivated cherry, plum, peach, apricot and wild cherry (Scortichini et al., 2003; Vicente and Roberts, 2007; Renick et al., 2008; Gilbert et al., 2009; Kaluzna et al., 2010b). There are three stone fruit diseases caused by *P. syringae* pathovars: *syringae*, *morsprunorum* and *persicae*. The pathovars *syringae* and *morsprunorum* are widely spread in our country, while the presence of pv. *persicae* has not been recorded and it is on the national A1 list of quarantine pathogens.

Both *P. s.* pv. *syringae* and pv. *morsprunorum* may cause bacterial canker of stone fruits: *P. s.* pv. *syringae* may cause canker on any stone fruit grown commercially, including pome fruits and herbaceous plants (Arsenijević, 1997; Gavrilović, 2006, 2009; Gavrilović et al., 2008), while *P. s.* pv. *morsprunorum* predominantly infects sour and sweet cherry, plum (Hattingh and Roos, 1995) and apricot (Bultreys and Kaluzna, 2010). Based on bacteriophage typing, two races of pv. *morsprunorum* have been described: race 1 (Wormald, 1932) pathogenic to cherry, plum and apricot, and race 2 (Freigoun and Crosse, 1975) that infects cherry (Bultreys and Kaluzna, 2010). The type of disease symptoms depends on a cultivar, age of the infected tree, plant tissue invaded, strain of the pathogen, and nature of the predisposing factors. Cankers develop on twigs at the base of flowers and leaf buds, in pruning wounds and at the base of infected spurs. Gum often exudes from cankers, especially early in the growing season. Terminal shoots or twigs of cankered trees may show dieback symptoms. The pathogens may be present in dormant leaf and flower buds, causing their necrosis. Some infected buds open in the spring, but collapse in early summer. Leaves that develop from these buds wilt, and fruit tends to dry out (Hattingh and Roos, 1995).

Bacterial decline caused by *P. s.* pv. *persicae* has been recorded on nectarine and peach in France, nectarine, peach and Japanese plum (*Prunus salicina*) in New Zealand and on *Prunus cerasifera* in the United Kingdom (OEEP/EPPO, 2005). On nectarine and peach, the symptoms include shoot dieback, limb and root injury, leaf spots and fruit lesions, and eventually death of the tree. On Japanese plum, the symptoms are mainly confined to dieback, occasional limb death, and leaf spots (Young, 1995). Dieback of terminal shoots can occur already in autumn, and in spring following the development of girdling lesions from nodal infections. Small elliptical lesions may develop at internodes. The rootstock can also be infected, showing symptoms similar to those on woody shoots. Leaf infection results in small, angular, water-soaked spots, while diseased tissue within the spots becomes brown, necrotic and subsequently falls out, causing the "shot hole" effect. On fruits, small, round, dark, oily spots occur. These can be spread within the fruit tissue, causing sunken, fruit deforming lesions with gum oozing out (OEEP/EPPO, 2005).

Some symptoms of bacterial decline can be confused with those of bacterial canker. Distinctive characteristics of decline are the staining of wood in branches above the necrosis and absence of an obvious boundary between the diseased and healthy bark in lower parts of the tree (Hattingh and Roos, 1995).

Due to their overlapping host range, symptomatology, and common bacteriological characteristics, *P. s.* pvs. *syringae*, *morsprunorum* and *persicae* can easily be misidentified. This may have significant consequences considering the quarantine status of pv. *persicae* in our country. The aim of this research was to study comparatively the characteristics of three pathovars using standard laboratory and pathogenicity tests as well as molecular PCR-based techniques, in order to determine appropriate methods for their rapid and efficient differentiation.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Reference strains of *P. s.* pv. *morsprunorum* KFB 0101 (LMG 5051t$_1$), *P. s.* pv. *persicae* KFB 0102 (LMG 5154) and *P. s.* pv. *syringae* KFB 0103 (LMG 1247), as well as *P. s.* pv. *morsprunorum* KFB 0120 were used in this study. The strains were stored in nutrient broth (NB) supplemented with 30% glycerol at -80°C. Prior to testing, bacterial cultures were grown on nutrient agar (NA) plates at 27°C for 24 h unless otherwise indicated.

Physiological and biochemical differentiation of pathovars

Gram reaction of the strains was determined using 3% KOH (Suslow et al., 1982, cited Arsenijević, 1997). Fluorescence on King's medium B was observed under UV light after 24-48 h of incubation (Schaad et al., 2001). Oxidative-fermentative (O/F) metabolism of glucose was performed using Hugh-Leifson medium (1953, cited Lelliott and Stead, 1987).

Although all three pathovars belong to the LOPAT group Ia (+ – – – +), levan production (L), oxidase activity (O), pectolytic capability (P), arginine dihydrolase (A) and tobacco hypersensitivity (T) were preformed in order to check potential differences in the intensity of reactions (Lelliott and Stead, 1987; Arsenijević, 1997; Schaad et al., 2001).

GATTa tests, consisting of gelatine hydrolysis (G), aesculin hydrolysis (A), tyrosinase activity (T) and utilization of tartaric acid (Ta), were carried out as

described by Jones (1971) and Lelliott and Stead (1987). The following tests were also used for pathovar differentiation: acid production from inositol, sorbitol and sucrose using the basal medium of Ayers et al. (1919, cited Lelliott and Stead, 1987), bacterial growth in sucrose nutrient broth (SNB) (Jones, 1971), ice nucleation (Lindow, 1990; Schaad et al., 2001), strains vitality on nutrient sucrose agar (NSA) and catalase activity (Arsenijević, 1997).

Detection of toxin-producing genes by Polymerase Chain Reaction (PCR)

The pathovars of *P. syringae* associated with stone fruits produce several well-characterized phytotoxic compounds which can be used for pathovar differentiation. The PCR method was used to detect genes involved in syringomycin synthesis (*syrB*), syringomycin secretion (*syrD*) and coronatine production (*cfl*).

For the detection of *syrB* gene, a set of primers (B1/B2) and a PCR procedure developed by Sorensen et al. (1998) were used with a reduction of final sample volume and volume of DNA. PCR amplification of the target sequence was performed in 50 µl of the following reaction mixture: 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$), 0.5 µM of each primer, 0.2 mM deoxynucleoside triphosphate (each), 1.25 U *Taq* DNA polymerase. As a template, 2 µl of bacterial suspension in sterile distilled water, conc. 10^8 CFU/ml (OD_{600} = 0.3) was used. The PCR steps were: template denaturation at 94°C for 1.5 min, primer annealing at 60°C for 1.5 min and DNA extension for 3.0 min at 72°C. After completing 35 cycles, an additional extension for 10 min at 72°C was included.

Detection of the *syrD* gene was carried out by the method of Bultreys and Gheysen (1999). Reaction mixtures (50 µl) contained 1×PCR buffer, 1.25 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (each), 25 pmol of each primer (SyD1/SyD2), 0.5 U *Taq* DNA polymerase and 2 µl of template DNA prepared by heating bacterial suspensions (approx. 10^8 CFU/ml) at 95°C for 10 min. Amplification was initiated by incubation at 93°C for 3 min, followed by 36 cycles at 93, 60, and 72°C for 1 min at each temperature and final extension at 72°C for 6 min.

The *Cfl* gene involved in coronatine synthesis was detected by the PCR method described by Bereswill et al. (1994). PCR mixtures were prepared in a final volume of 25 µl: 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8) and 1.5 mM $MgCl_2$), 0.8 µM of each primer (Primer 1/Primer 2), 0.2 mM deoxynucleoside triphosphates (each), 1.5 U *Taq* DNA polymerase, and 1 µl of bacterial suspension in sterile distilled water (approx. 10^7 CFU/ml). PCR cycling parameters consisted of an initial denaturation step at 93°C for 2 min, followed by 37 cycles of 2 min at 93°C, 1 min at 67°C and 2 min at 72°C.

Primer names, oligonucleotide sequences, the expected size of amplified products, and literature sources are listed in table 1. PCR reactions were performed in a Thermo Cycler 2720 (Applied Biosystem, USA). The PCR products (5 µl) were separated by agarose gel (1%) electrophoresis in Tris-acetate-EDTA (TAE) buffer, stained in ethidium bromide (1 µg/ml) and visualized under UV light by a digital imaging camera (Vilber Lourmat, France).

Table 1. Primers used in PCR methods for *Pseudomonas syringae* pathovar differentiation

Primer	Sequences	Size of amplified product	References
B1/ B2	5'-CTTTCCGTGGTCTTGATGAGG-3' 5'-TCGATTTTGCCGTGATGAGTC-3'	752 bp	Sorensen et al., 1998.
SyD1/ SyD2	5'-CAGCGGCGTTGCGTCCATTGC-3' 5'-TGCCGCCGACGATGTAGACCAGC-3'	1040 bp	Bultreys and Gheysen, 1999.
Primer1/ Primer2	5`-GGCGCTCCCTCGCACTT-3` 5`-GGTATTGGCGGGGGTGC-3`	650 bp	Bereswill et al., 1994.
Rep-PCR			
REP1R-I/ REP2-I	5'-IIIICGICGICATCIGGC-3' 5'-ICGICTTATCIGGCCTAC-3'	/	Schaad et al., 2001.
ERIC1R/ ERIC2	5'-ATGTAAGCTCCTGGGGATTCAC-3' 5'-AAGTAAGTGACTGGG GTGAGCG-3'	/	
BOXAIR	5'-CTACGGCAAGGCGACGCTGACG-3'	/	

Differentiation by Rep-PCR

All strains were subjected to Rep-PCR genomic fingerprinting using primer sets corresponding to REP, ERIC, and BOX elements (Table 1) (Schaad et al., 2001). Template DNA was prepared by heating bacterial suspensions (approx. 10^8 CFU/ml) at 95°C for 10 min followed by incubation on ice for 5 min. The Rep-PCR reaction mixture consisted of 1 x PCR Master mix (Fermentas, Lithuania), 4 mg bovine serum albumin (BSA), 10% dimethylsulfoxide (DMSO), 3 μM of each primer and 1 μl of DNA template. Sterile distilled water was added to the final volume of 25 μl. PCR amplification reactions were performed in a Thermo Cycler 2720 (Applied Biosystem, USA) using the following conditions: an initial denaturation at 95°C for 2 min; 35 cycles consisting of 94°C for 3 s and 92°C for 30 s; annealing at 40°C for 1 min for REP primers or at 50°C for ERIC and BOX primers; extension at 65°C for 8 min; and final extension cycle at 65°C for 15 min before cooling at 4°C. The amplified PCR products were resolved by 1.5% agarose gel electrophoresis in TAE buffer and visualized on an UV transilluminator (Vilber Lourmat, France) after staining in ethidium bromide (1 μg/ml) solution.

Syringomycin bioassay

Reference strains of *Pseudomonas syringae* pvs. were streaked in a straight line onto Potato Dextrose Agar medium (PDA) and grown for 3-4 days at 27°C. Meanwhile, the cultures of syringomycin sensitive organisms, *Geotrichum candidum, Saccharomyces cerevisiae* and *Rhodotorula pilimanae*, were cultivated for 3-4 days under the same conditions. The surface of the medium, containing 4-day-old bacterial culture, was sprayed with a suspension of the indicator organism in sterile distilled water. After further incubation for 1-2 days, clear zones of fungi growth inhibition were observed around bacterial colonies as an indication of syringomycin production.

Pathogenicity tests

For inoculum preparation, 24 h-old cultures of the tested strains grown on King's medium B were suspended in sterile distilled water and adjusted to approx. 10^8 CFU/ml (OD_{600} = 0.3). The suspension was injected with a syringe and hypodermic needle into string bean pods, infiltrating a tissue area of approx. 10 mm in diameter. Sterile distilled water was used as a negative control. Inoculated string bean pods were incubated at high humidity conditions at room temperature. Necrosis of the infiltrated tissues was observed after 24-48 h.

In addition to hypersensitive reaction of tobacco (within LOPAT) and string bean pods, pathogenicity of the strains was studied by inoculating lemon and immature nectarine fruits. The fruits were prick-inoculated with a hypodermic needle, leaving a droplet of bacterial suspension (10^8 CFU/ml) at the inoculation site. In order to maintain high humidity, inoculated fruits were placed on wet filter paper in a sealed plastic container and maintained at room temperature. Development of symptoms was observed daily during seven days.

RESULTS

Physiological and biochemical characteristics

All studied *Pseudomans syringae* pathovars were Gram-negative and belonged to LOPAT Group Ia of the determinative scheme of Lelliott et al. (1966) (Table 2). Although all three pathovars produced mucoid "levan-type" colonies on NSA medium, differences were observed in their appearance and size (Figure 1). *P. s.* pv. *persicae* formed small colonies, 1-2 mm in diameter, compared to the other two pathovars that formed colonies 3-4 mm in diameter after three days of incubation. *P. s.* pv. *persicae* grew more slowly on King's medium B and did not produce green fluorescent pigment as the other two pathovars. Unlike pv. *persicae*, pvs. *syringae* and *morsprunorum* hydrolyzed gelatin and aesculin. Pathovar *morsprunorum* produced tyrosinase, while none of the tested pathogens utilized tartaric acid.

P. s. pv. *morsprunorum* did not show ice nucleation activity, while the other two pathovars were positive. The same pathovar exhibited white growth in SNB medium, while pv. *syringae* produced yellow growth. According to vitality test on NSA medium, pv. *morsprunorum* lost its vitality and showed negative catalase reaction after 4 days, while the other two pathovars remained vital for at least seven days. All tested strains showed oxidative metabolism of glucose. Pvs. *syringae* and *morsprunorum* produced acid from inositol, sorbitol and sucrose, while pv. *persicae* produced acid from sorbitol and sucrose but not from inositol.

Table 2. Results of biochemical and physiological tests of the studied *Pseudomonas syringae* pathovars

Test	*Pseudomonas syringae*		
	pv. *syringae* KFB 0103	pv. *morsprunorum* KFB 0101	pv. *persicae* KFB 0102
Gram reaction	-	-	-
Fluorescence on King's medium B	+	+	-
Levan production (L)	+	+	+
Oxydase reaction (O)	-	-	-
Pectolytic capability (P)	-	-	-
Arginine dihydrolase (A)	-	-	-
Tobacco hypersensitivity (T)	+	+	+
Gelatine hydrolysis (G)	+	+	-
Aesculin hydrolysis (A)	+	+	-
Tyrosinase activity (T)	-	+	nt
Utilization of tartaric acid (Ta)	-	-	-
Acid production from:			
Inositol	+	+	-
Sorbitol	+	+	+
Erytritol	+	+	+
Ice nucleation	+	-	+
O/F test	O	O	O
Nutrient sucrose broth growth	yellow	white	nt
Vitality on NSA medium	7 days	4 days	7 days

Legend: + - positive reaction, – - negative reaction, nt - not tested, O - oxidative metabolism.

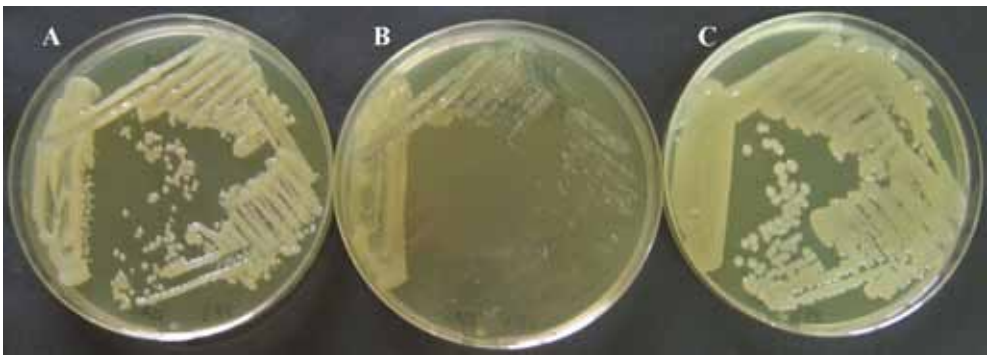

Figure 1. "Levan-type" colonies on NSA medium. A - *P. s.* pv. *morsprunorum* (KFB 0101), B - *P. s.* pv. *persicae* (KFB 0102), C - *P. s.* pv. *syringae* (KFB 0103)

Molecular characterization

PCR methods were used for detection of toxin encoding genes: *syrB*, *syrD* and *cfl*. The genes for syringomycin synthesis (*syrB*) and syringomycin secretion (*syrD*) were detected in *P. s.* pv. *syringae* strain KFB 0103 by amplifying DNA fragments of 752 bp and 1040 bp, respectively (Figure 2A and 2B). Coronatine production gene (*cfl*) was detected in *P. s*. pv. *morsprunorum* strain KFB 0120, which belongs to race 1 and produced an amplicon of 650 bp (Figure 2C). Rep-PCR protocol using REP, ERIC and BOX primers revealed differences in the genetic profiles of the tested pathovars (Figure 3).

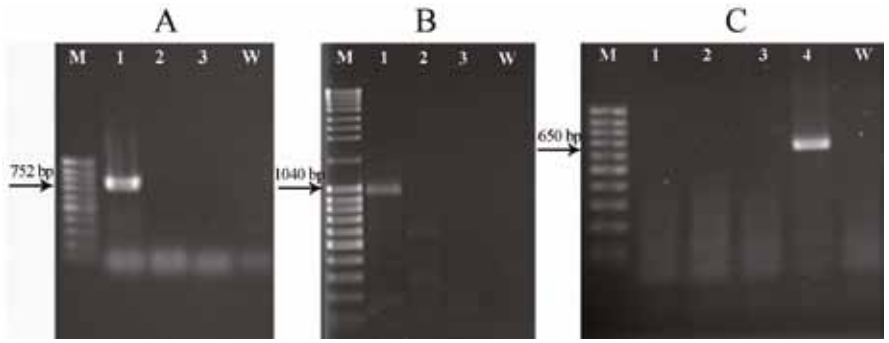

Figure 2. PCR detection of genes involved in phytotoxins production: *syrB* (A), *syrD* (B) and *cfl* (C). M – marker (MassRuler Low Range (A and C) and Mix Range (B) DNA Ladder, Fermentas, Lithuania), 1 – *P. s.* pv. *morsprunorum* (KFB 0101), 2 – *P. s.* pv. *persicae* (KFB 0102), 3 – *P. s.* pv. *syringae* (KFB 0103), 4 – *P. s.* pv. *morsprunorum* (KFB 0120), W – negative control

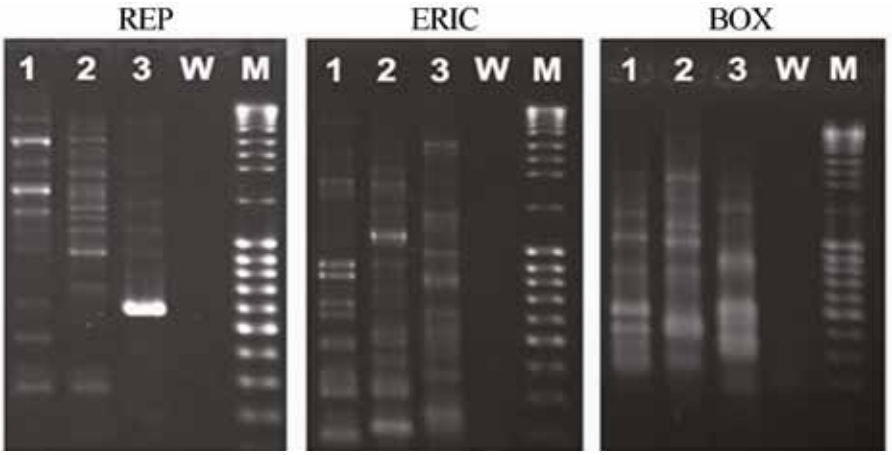

Figure 3. Rep-PCR fingerprinting patterns from genomic DNA of *Pseudomonas syringae* strains. REP-PCR, ERIC-PCR and BOX-PCR patterns are shown. 1 – *P. s.* pv. *syringae* (KFB 0103), 2 – *P. s.* pv. *morsprunorum* (KFB 0101), 3 – *P. s.* pv. *persicae* (KFB 0102), W – negative control, M – marker (MassRuler Low Range DNA Ladder, Fermentas, Lithuania)

Figure 4. Inhibition zone (↑) of *Saccharomyces cerevisiae* (A) and *Rhodotorula pilimanae* (B) growth on PDA medium caused by *P. s.* pv. *syringae* strain KFB 0103

Syringomycin bioassay

Bioassay for syringomycin production on PDA medium showed that *P. s.* pv. *syringae* strain KFB 0103 produces this toxin as indicated by a zone of inhibition of *Geotrichum candidum, Saccharomyces cerevisiae* and *Rhodotorula pilimanae* growth (Figure 4).

Pathogenicity tests

Pathogenicity of *P. s.* pvs. *syringae*, *morsprunorum* and *persicae* was confirmed by hypersensitive reaction of tobacco leaves and string bean pods, as well as by inoculation of lemon and immature nectarine fruits. Differences in symptom severity were observed among the three *P. syringae* pathovars (Figure 5). Positive HR response on string bean pods was visible after 24-48 h: brown tissue necrosis developed at the site of bacterial suspension infiltration. We observed that tissue infiltrated with pv. *syringae* developed a more intensive necrosis than the pods infiltrated with the other two pathovars (Figure 5A). Lemon and immature nectarine fruits also showed necrosis of tissue at the inoculation site seven days after inoculation. More intensive necrotic lesions were recorded on nectarine and lemon fruits inoculated with pv. *syringae* compared with the other two pathovars (Figure 5B and 5C).

DISCUSSION

P. s. pv. *syringae* and pv. *morsprunorum* have been widely distributed stone fruits pathogens in Serbia, unlike the quarantined pv. *persicae*, recorded only in France, New Zealand and the UK (OEEP/EPPO, 2005). Due to similarity in the host range, symptomatology and physiological and biochemical characteristics, these pathogens can be easily misidentified. In order to select differential tests for rapid and reliable identification of *P. s.* pvs. *syringae*, *morsprunorum* and *persicae*, we studied their pathogenicity, physiological and biochemical characteristics, the presence of genes for toxin production, as well as differences in the genetic profile.

Gram reaction, fluorescence on King's medium B and LOPAT tests are standard tools for separating *Pseudomonas* spp. from plant pathogenic bacteria belonging to other genera as well as for partial differentiation of *P. syringae* species from some pseudomonads. However, additional biochemical tests should be used for discrimination of *P. syringae* pathovars (Table 2). Although growth of bacteria on KB medium and production of green fluorescent pigment are used as a general test for differentiation of *Pseudomonas* sp. from bacteria belonging to other genera, it can also be indicative of pv. *persicae*. In our study *P. s.* pv. *persicae* grew more slowly on this medium than the other two pathovars and did not produce green fluorescent pigment.

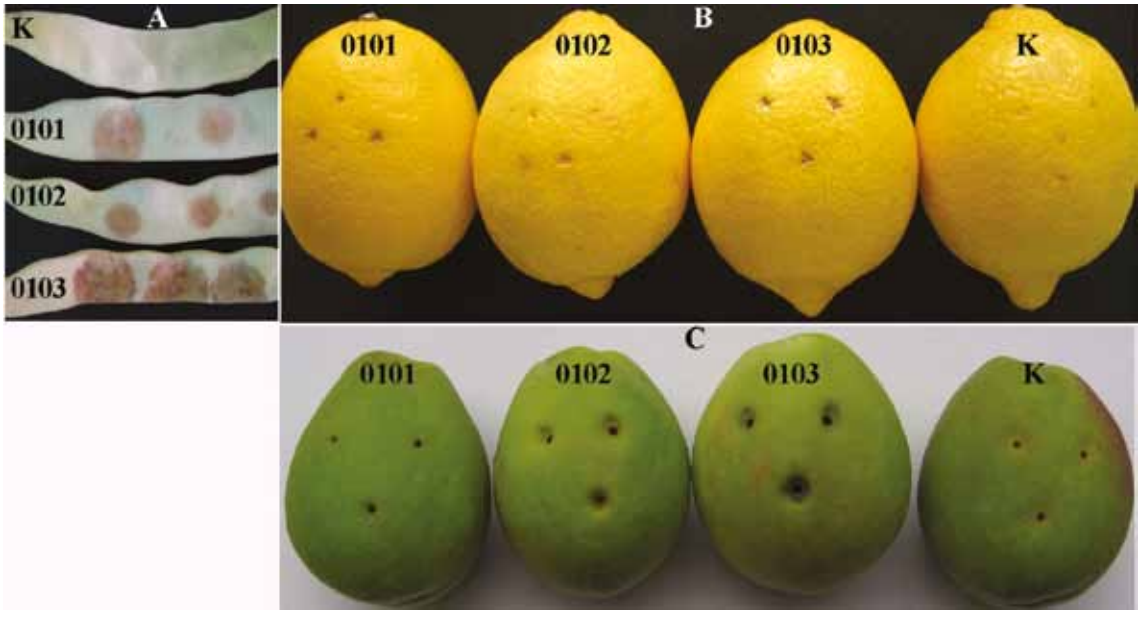

Figure 5. Pathogenicity test on string bean pods (A), lemon fruits (B) and nectarine fruits (C). 0101 – *P. s.* pv. *morsprunorum,* 0102 – *P. s.* pv. *persicae*, 0103 – *P. s.* pv. *syringae*, K – negative control

However, this characteristic is not entirely discriminative since nonfluorescent pv. *morsprunorum* strains have recently been isolated (Bultreys and Kaluzna, 2010). *P. s.* pv. *persicae* also produced less growth and smaller "levan type" colonies on NSA medium compared to the other two pathovars (Figure 1). The same medium was used for strains differentiation according to their vitality, i.e. catalase activity. Although it takes 4-6 days for the results, this test is simple to perform and could be recommended as differential.

The GATTa tests have been shown to be reliable only for discrimination of pvs. *syringae* and *morsprunorum* race 1 (Latorre and Jones, 1979), while the strains of *P. s.* pv. *morsprunorum* race 2 are variable in these tests (Gilbert et al., 2009). Strain KFB 0101, used in this study, belongs to *P. s.* pv. *morsprunorum* race 2, which hydrolyzed gelatine and aesculin, produced tyrosinase and did not utilize tartaric acid (Table 2). As reported in literature, results of GATTa tests for *P. s.* pv. *morsprunorum* race 1 strains are homogeneous: they do not hydrolyze gelatin and aesculin but produce tyrosinase and utilize tartaric acid (Bultreys and Kaluzna, 2010). Therefore, only gelatine hydrolysis can be used as a reliable test for pv. *morsprunorum* race differentiation in GATTa tests since it is negative for race 1 and positive for race 2. Vicente and Roberts (2007) showed that some *P. s.* pv. *morsprunorum* race 2 strains could give the same GATTa tests results (+ + − −) as pv. *syringae* strains. In addition, the results from the GATTa tests for other *P. syringae* pathovars (more than 50 known pathovars) are largely unknown and therefore it is possible that they could give the same GATTa results as pv. *syringae* strains. However, we recommend using GATTa tests preferably for differentiation of pvs. *syringae* and *morsprunorum* race 1 and as an additional test for pv. *morsprunorum* race 2 identification. In addition to GATTa, other biochemical tests, such as ice nucleation, bacterial growth in sucrose nutrient broth and acid production from inositol can be used as differential (Table 2). These tests are rapid, reliable, inexpensive and simple to perform. The pathogenic variety *morsprunorum* can be differentiated from the other two pathovars by its inability to initiate ice nucleation, while acid production from inositol separates pv. *persicae.* Yellow and white growth of strains in SNB medium can be used for pvs. *syringae* and *morsprunorum* race 1 differentiation but not for *morsprunorum* race 2 since results for this race can vary (Bultreys and Kaluzna, 2010).

Hypersensitive reaction of tobacco leaves is a reliable indication of the pathogenic nature of the tested bacterium. However, it is not a substitute for pathogenicity testing by inoculating susceptible host plant. In case of these pathovars, the development of typical symptoms on woody tissue of stone fruit plants can take more than a month. Therefore, our intention was to find other plant material generally available and suitable for testing of *Pseudomonas* pvs. pathogenicity. Immature string bean pods could be used as a suitable alternative since the pathovars consistently differed in severity of reaction. Two days after inoculation, pv. *syringae* caused the most intensive necrosis of the infiltrated tissue, compared to the other two pathovars. Besides tobacco plants and bean pods, we inoculated lemon and immature nectarine fruits as well. Symptoms on these plant organs appeared within seven days. A slight difference in symptoms was observed among the three pvs., but they were barely noticeable and not reliable for differentiation (Figure 5). In addition to plants used in this study, pathogenicity test can also be performed on lilac leaves and the pathogenic reaction can be assessed 14 days after inoculation (Young, 1991). Gavrilović et al. (2008) used cherry, pear, tomato and pepper fruits and pear seedlings for *P. s.* pv. *syringae* and pv. *morsprunorum* pathogenicity testing. In those tests, first symptoms were observed 24h after inoculation on cherry fruits, while symptoms on other plant organs appeared three days later. The choice of plant organs selected for pathogenicity testing will also depend on their availability.

P. syringe pathovars associated with stone fruits differ in their production of toxins. Therefore, this feature can be used for their identification, either by direct detection of these secondary metabolites or by detection of a gene involved in toxin production or secretion. The primers for detection of *syrB* or *syrD* gene involved in the synthesis and secretion of syringomycin, respectively, efficiently differentiated pv. *syringae*, while the primers for *cfl* gene detection, involved in coronatine production, differentiated pv. *morsprunorum* from the other two pathovars. However, phytotoxin test results are relevant only in the case of a positive result because phytotoxin production is not constant in pv. *syringae* and pv. *morsprunorum* race 1 (Bultreys and Kaluzna, 2010). Gilbert et al. (2009) reported that in pv. *morsprunorum* race 1, strains indistinguishable by BOX-PCR may be positive or negative for coronatine production. However, the situation is different with pv. *syringae* because the toxin-negative strains from pear identified as pv. *syringae* by the GATTa tests were shown to differ genetically from the toxin-positive ones (Gilbert et al., 2009).

Rep-PCR has been the most commonly used molecular method for analyzing the diversity of pathogens causing stone fruit bacterial diseases. This is a rapid test for differentiating strains of the two races of pv. *morsprunorum* because of the homogeneity found in each race. Also, it can be useful as a supplementary test for pv. *syringae* identification (Menard et al., 2003; Vincente and Roberts, 2007). Recent studies on these methods have confirmed that there are homogeneities in different DNA regions of each pv. *morsprunorum* race and high diversity among pv. *syringae* strains (Gilbert et al., 2009; Kaluzna et al., 2010a).

In addition to tests used in this study, there are other methods available for *P. syringae* pathovars detection and identification, such as serological agglutination and ELISA tests (Burkowicz and Rudolph, 1994; Vicente et al., 2004). The advantages of these techniques are that they are less time consuming, simple and robust, and have possibilities for screening many samples and for automation. A disadvantage of ELISA is its low detection level (10^5-10^6 cells/ml). Both agglutination and ELISA have the disadvantage of showing disturbing cross-reactions of non-target bacteria with antisera used (Janse, 2005). Bacteriophage typing was used for detection and differentiation of pv. *morsprunorum* race 1 and pv. *syringae*. By this method, Crosse and Garrett (1963, 1970) divided isolates of pv. *morsprunorum* race 1 from cherry and plum into subtypes; the distinction between isolates was related to host specificity in the field. Multilocus Sequence Typing (MLST) based on housekeeping gene analysis, has also been used for characterizing and differentiating stone fruit *P. syringae* strains. This method revealed high discrimination among *P. syringae* isolates (Sarkar and Guttman, 2004; Hwang et al., 2005; Kaluzna et al., 2010a). Although the MLST procedure is a powerful and highly discriminatory method for analysing pathogen population structure and epidemiology, there are some disadvantages that include the requirements for specialized equipment, expensive reagents, highly trained personnel and the fact that the method may not be amenable to the analysis of all microorganisms. Low sequence diversity may preclude its usefulness to distinguish between isolates and makes it more difficult to accurately assess the genetic relatedness between isolates.

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Diferencijacija *Pseudomonas syringae* patogenih varijeteta poreklom iz koštičavih voćaka

REZIME

Patogeni varijeteti *Pseudomonas syringae* poreklom sa koštičavih voćaka poseduju brojne zajedničke karakteristike u pogledu kruga domaćina, simptomatologije i biohemijsko-fizioloških osobina, što otežava njihovu identifikaciju. U cilju odabira testova pogodnih za brzu i pouzdanu identifikaciju *P. s.* pv. *syringae*, *morsprunorum* i *persicae*, primenjeni su standardni bakteriološki i molekularni testovi. Diferencijacija sojeva izvršena je LOPAT i GATTa testovima, posmatranjem razvoja u hranljivom rastvoru sa saharozom, sposobnošću sojeva da formiraju čestice leda, kao i mogućnošću korišćenja različitih ugljenikovih jedinjenja. PCR metod korišćen je u detekciji gena odgovornih za proizvodnju toksina siringomicina kod soja *P. s.* pv. *syringae (syrB* i *syrD* geni) i koronatina kod soja *P. s.* pv. *morsprunorum* rase 1 (*cfl* gen). Proizvodnja siringomicina potvrđena je i biotestom, korišćenjem gljiva *Geotrichum candidum, Saccharomyces cerevisiae* i *Rhodotorula pilimanae* kao indikatora. Proverom patogenosti sojeva na plodovima limuna, nesazrelim plodovima nektarine i mahunama boranije, došlo je do ispoljavanja simptoma različitog intenziteta, na osnovu kojih se može izdvojiti pv. *syringae* od ostala dva patovara. Primenom Rep-PCR metode, uz korišćenje REP, ERIC i BOX prajmera, ustanovljene su razlike u genetskim profilima proučavanih *P. syringae* patogenih varijeteta.

Ključne reči: *Pseudomonas syringae*; koštičavo voće; identifikacija; PCR