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DI[illegible]TY AMONG *PSEUDOMONAS SYRINGAE* STRAINS [illegible]GINATING FROM FRUIT TREES IN SERBIA

Ž. IVANC[illegible]VETLANA ŽIVKOVIĆ[1], MIRA STAROVIĆ[1], DRAGANA JOŠIĆ[2], S. STANKOVIĆ[3], and V. GAVRILOVIĆ[1]

[1]*I*[illegible] *Plant Protection and the Environment,* 11000 Belgrade, Serbia

[2]*Soil Institute,* 11000 Belgrade, Serbia

[illegible]*Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia

Abstract — *Pseudomonas syringae* is a widespread and economically important plant pathogen, one found on a number of hosts, including fruit trees, field crops, vegetables, and ornamental plants. This bacterium has been experimentally identified as a parasite of pear, apple, apricot, peach, cherry, sour cherry, plum, and raspberry. The present study was designed to establish differences between strains isolated from fruit trees in Serbia. The pathogenic and biochemical characteristics of isolates were studied. The BOX-PCR method was used to generate genomic fingerprints of *Pseudomonas syringae* isolates and to identify strains that were previously not distinguishable by other classification methods. Different *Bacillus* sp. strains were tested for in vitro inhibitory activity against *Pseudononas syringae* isolates. *Bacillus* sp. strains show inhibitory activity only against *P. syringae* isolates that originated from peach. The obtained results demonstrate that the population of the bacterium *Pseudomonas syringae* from the fruit trees in Serbia is very diverse.

Key words: *Pseudomonas syringae*, *Bacillus* sp., fruit trees, BOX-PCR, pathogenicity, biochemical properties, diversity

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INTRODUCTION

The genus *Pseudomonas* contains Gram-negative bacteria that are asporogenous, rod-shaped, with mono- and peritrichous flagellation, and with respiratory glucose metabolism (Doudoroff and Palleroni, 1984). They are often found in soil and water, where they have a role in decomposing organic material. Some species are well-known as plant pathogens and cause symptoms of blossom blast, shoot blight, bud necrosis, branch decay, dying of whole trees, etc. (Gavrilović, 2004). The most widespread and economically most important plant pathogen is *Pseudomonas syringae*, which is found on a number of hosts, including fruit trees, field crops, vegetables, and ornamental plants (Arsenijević, 1997).

Recently, *Pseudomonas syringae* has emerged as a fruit tree pathogen causing significant economic losses in Serbia. This bacterium has been experimentally identified as a parasite of pear, apple, apricot, peach, cherry, sour cherry, plum, and raspberry, causing blossom blast and shoot blight, as well as necrosis of buds, fruits, and branches (Josifović and Šutić, 1964; Arsenijević, 1968, 1970; Radman, 1969; Arsenijević and Balaž, 1986; Arsenijević and Sremac, 1993; Gavrilović, 2004; Gavrilović et al., 2004, 2008, 2009).

According to the established taxonomy, *P. syringae* on fruit trees is represented with two varieties: pv. *syringae*, found on pome and stone fruits; and pv. *morsprunorum*, found on stone fruits only, especially cherry and sour cherry (Latore and Jones 1979; Arsenijević, 1997). Intermediate strains exhibiting characteristics of both varieties were isolated from necrotic sour cherry fruits (Sobiczewski, 1984; Balaž et al., 1988). In addition, a new pathogen variety (*avii*) was detected on cherry trees in France using molecular methods (Menard et al., 2003).

There is a need for further study of the epidemiology of *P. syringae* with the accent on prevention and control of further spreading of the diseases caused by this pathogen. In order to do this successfully, it is crucial to ensure accurate identification of the bacterium by applying bacteriological and molecular methods.

Pathovars within each species cannot be reliably distinguished by their cellular metabolism or other phenotypic characteristics (Van Zyl et al., 1990; Stead et al., 1992). They are therefore classified on the basis of their distinctive pathogenicity to one or more host plants (Young et al.,1991). Scortichini et al. (2003) used different primers to establish the difference between varieties from different pome fruit trees and even among strains isolated from the same hosts. These results showed significant differences among the varieties of *P. syringae*. We presumed that use of rep-PCR-based technique should enable us to generate unique genomic fingerprints for each pathovar of the pathogen.

The aim of this work was to establish differences between strains isolated from fruit trees in Serbia using BOX PCR and learn which pathovars are present in Serbia. Another aim was to establish the taxonomic status of strains of *P. syringae* originating from diseased sour cherry and raspberrry fruit. In addition, we tested the antagonistic effect of *Bacillus* sp. on *Pseudomonas syringae* isolates.

MATERIALS AND METHODS

Bacterial strains

Strains of *Pseudomonas syringae* were isolated between 2004 and 2008 from pear, peach, apple, plum, cherry, sour cherry, and raspberry plants from different localities in Serbia (Table 1).

The strains CFBP 11 (*P. s.* pv. *syringae*), CFBP 1582 (*P. s.* pv. *syringae*) and CFBP 2119 (*P. syringae* pv. *morsprunorum*) from the French collection of phytopathogenic bacteria were used in this work as references.

Pathogenicity tests

Pathogenic characteristics of the isolates were tested by artificial inoculation of pear, cherry, and lemon fruit, lilac leaves, and bean pods using the procedure described by Klement (1990).

In order to check the hypersensitive reaction (HR), tobacco and *Geranium* leaves were inoculated with a bacterial suspension of 107 cfu/ml (Klement, 1963).

Bacteriological characteristics

The following morphological, cultivation, and biochemical characteristics were studied: Gram differentiation of bacterial isolates was performed according to the rapid method described by Suslow et al. (1982). Production of fluorescent pigment on King's medium B (King, 1954) was carried out according to Lelliott et al. (1966). Glucose metabolism was tested according to standard protocol (Hugh and Leifson, 1953).

Pseudomonas syringae strains were subjected to LOPAT tests for levan production (Lelliott et al., 1966), the cytochrome oxidase reaction (Kovacs, 1956), and arginine dehydrogenase and pectinase activity (Thornley, 1960).

In addition to these characteristics, the following biochemical tests were carried out to differentiate between pv. *syringae* and pv. *morsprunorum*: for gelatin and esculin hydrolysis; for tyrosinase activity; and for tartrate metabolism (GATT) (Arsenijević, 1997).

Isolation of DNA

Total genomic DNA was prepared using a modification of the procedure of Ausubel et al. (1992). Cultures were grown in SNA (sucrose nutrient agar) medium for 48 h at 25°C. Bacterial cells were rinsed with sterile distilled water and centrifuged at 4,000 × g for 10 min at 4°C. The pellet was resuspended twice in 0.85% NaCl and once in 0.1 M $NaPO_4$ buffer (pH 6.8). Cells were treated with 10% sodium dodecyl sulfate (SDS) and mixed with 20 mg of proteinase K per ml at 37°C for 1 h. Sodium chloride was added to a final concentration of 5 M, and DNA was purified using a solution of 10% hexadecyltrimethyl ammonium bromide (CTAB) in 1 M NaCl at 65°C for 10 min, followed by phenol-chloroform and chloroform extractions. The DNA was recovered by isopropanol precipitation, redissolved in Tris-EDTA (TE, 10 mM Tris, 1 mM EDTA, pH 8.0), and quantified spectrophotometrically at 260 nm.

Table 1. Isolate number, host, locality, and year of isolation of *Pseudomonas syringae* strains.

STRAIN	HOST	LOCALITY	YEAR OF ISOLATION
IZB-193	Apple	Bela Crkva	2007
IZB-26	Pear	Šabac	2008
IZB-8	Raspberry	Arilje	2006
IZB-29	Cherry	Šabac	2008
IZB-135	Sour cherry	Bela Crkva	2007
IZB-156	Plum	Šabac	2004
IZB-62	Peach	Smederevo	2008
CFBP-11*			
CFBP-1582*			
CFBP-2119*			

Amplification and separation of DNA bands

Amplification was performed in a total volume of 25 µl containing 67 mM Tris-HCl (pH 8.8); 25 mM $MgCl_2$; 125 µM of dATP, dCTP, dGTP, and dTTP each; 2 units of Taq DNA polymerase (Fermentas, Lithuania); and 100 pmol of BOXA1R primer. A 40-ng quantity of genomic DNA or distilled water as a negative control was added to the reaction tubes. The primer was a sequence corresponding to BOX A, a subunit of the BOX element (Lupski et al., 1992): BOXAlR [5'-CTAC GGCAAGGCGACGCTGACG-3']. The PCR conditions were as previously described (de Bruijn, 1992). The PCR protocols with BOX primer are referred to as BOX-PCR and rep-PCR collectively. Amplification of PCR was performed with a Mastercycler personal model (Eppendorf, Hamburg, Germany) using the following cycles: one initial cycle at 95°C for 7 min; 30 cycles of denaturation at 94°C for 1 min; annealing at 52°C for 1 min; and extension at 65°C for 8 min, with a single final extension cycle at 65°C for 16 min and a final soak at 4°C. Amplified PCR products were separated by gel electrophoresis on 1% agarose gels in 0.5 X TAE buffer for 2 h at 5 V/cm, stained with ethidium bromide, and visualized under UV illumination. Fingerprints generated from different strains were compared visually.

In vitro bioassay

Different *Bacillus* sp. strains were tested for the production of compounds inhibitory to *Pseudomonas syringae* strains as described elsewhere (Harris et al., 1989; Stanković et al., 2007). A strain was scored positive if a clear inhibiton zone of at least 2 mm in diameter was observed.

RESULTS

Pathogenicity

The investigated isolates showed significant heterogeneity of pathogenic characteristics. All of them caused hypersentivity (HR) on tobacco, but in other respects the isolates behaved differently.

The isolates from peach, pear, apple, and raspberry caused necrosis on inoculated unripe pear, cherry, and lemon fruits, lilac leaves, and bean pods, demonstrating typical characteristics of *P. syringae* pv. *syringae*.

The isolates from necrotic plum and cherry buds and sour cherry fruit caused necrosis of cherry fruit, but without effects in other tests, showing characteristics of *P. syringae* pv. *morsprunorum*.

On the basis of pathogenicity, the investigated isolates could clearly be divided into two groups: the first group isolated from peach, pear, apple, and raspberry, and the second one from sour cherry and plum.

Bacteriological characteristics

All investigated strains are Gram-negative and fluorescent on King's medium B; metabolize glucose aerobically; and produce levan, but not oxidase, arginine dehydrolase, or pectinase (Table 2).

Table 2. Biochemical characteristics of *P. syringae* strains from different fruit trees.

Strain	IZB-26	IZB-193	IZB-29	IZB-135	IZB-156	IZB-8	IZB-62	CFBP-11	CFBP1-582	CFBP-2119
Fluorescent	+	+	+	+	+	+	+	+	+	+
Glucose (O/F) metabolism	O	O	O	O	O	O	O	O	O	O
Levan production	+	+	+	+	+	+	+	+	+	+
Oxidase activity	-	-	-	-	-	-	-	-	-	-
Arginine dehydrolase	-	-	-	-	-	-	-	-	-	-
Pectinase activity	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	-	+	-	-	+	+	-	-
Esculin hydrolisis	+	+	-	-	-	+	+	+	+	-
Tyrosinase production	-	-	+	+	+	+	-	-	+	+
Tartrate metabolism	-	-	+	-	+	+	-	-	+	+

According to the results of biochemical tests for differentiation of pv. *syringae* and pv. *morsprunorum*, the investigated strains can be divided into three clearly distinct groups. The strains isolated from pear, peach, and apple were positive in gelatin and esculin hydrolisis, but negative in tyrosinase activity and metabolism of tartrate. In contrast, the strains from plum and cherry did not hydrolyze gelatin or esculin and were positive in the tyrosinase and tartrate tests. Isolated from raspberry and sour cherry, strains of the third group exhibited intermediate characteristics (Table 2).

Analysis of DNA

The previous classification (Latore and Jones, 1978), based solely on pathogenicity tests and biochemical characteristics, was not satisfactory since identified intermediate forms could not be classified as pv. *syringae* or pv. *morsprunorum*. With the aid of more advanced molecular techniques, new pathogenic varieties were identified (Menard et al., 2003).

The rep-PCR genomic fingerprints generated with BOX primer from the 10 virulent isolates enabled us to distinguish between the different strains of *Pseudomonas syringae*. The fingerprint patterns of *Pseudomonas syringae* strains are shown in Fig. 1. Differences among strains were assessed visually on the basis of migration patterns of the PCR products.

Fingerprint profiles generated with BOX primer were complex and very different between the isolates. The conducted BOX-PCR yielded 5 to 15 distinct PCR products, ranging in size from approximately 100 bp to over 6 kb. Differences among pathovars were assessed visually on the basis of the migration patterns of PCR products. Analysis of BOX-PCR clearly differentiated the pathovar reference strains of pv. *syringae* and pv. *morsprunorum* (Fig. 1, lane 2 versus lanes 3 and 4). Fragments of DNA generated from *Pseudomonas syringae* strains isolated from plum and cherry were similar to ones isolated

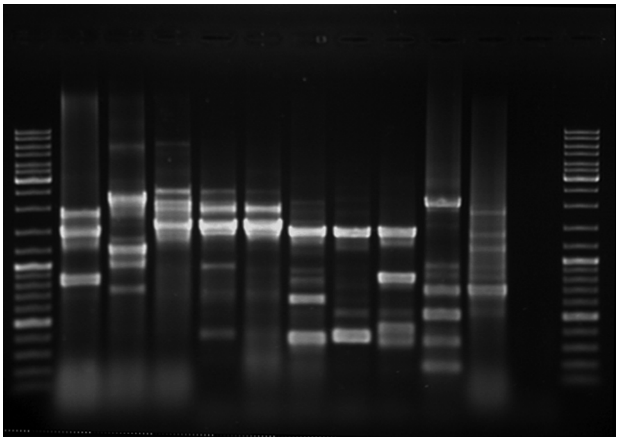

Fig. 1. Agarose gel electrophoresis of repetitive sequence-based polymerase chain reaction (BOX-PCR) fingerprint patterns obtained from *Pseudomonas syringae.* Lane 1) DNA molecular size marker (GeneRulerTM DNA Ladder Mix); lane 2) CFBP-11; lane 3) CFBP-1582; lane 4) CFBP-2119; lane 5) *P. syringae* isolate from plum; lane 6) *P. syringae* isolate from cherry; lane 7) *P. syringae* isolate from sour cherry; lane 8) *P. syringae* isolate from pear; lane 9) *P. syringae* isolate from apple; lane 10) *P. syringae* isolate from raspberry; lane 11) *P. syringae* isolate from peach; lane 12) negative control.

Table 3. Inhibitory activity of *Bacillus* sp. against *Pseudomonas syringae*.

Pseudomonas syringae strains	Inhibitory activity of *Bacillus* sp.
IZB-193	-
IZB-26	-
IZB-8	-
IZB-29	-
IZB-135	-
IZB-156	-
IZB-62	+

from the reference strain *Pseudomonas syringae* pv. *morsprunorum* (CFBP-2119) originating from cherry (Fig. 1, lanes 4 to 6). The BOX-PCR profiles of *P. syringae* strains isolated from sour cherry, pear, apple, raspberry, and peach were found to be different from all three reference strains (Fig. 1, lanes 7 to 11 versus lanes 2 to 4).

Different *Bacillus* spp. strains were tested for *in vitro* inhibitory activity against *Pseudononas syringae* isolates (Table 3). *Bacillus* spp. strains showed inhibitory activity only against *P. syringae* isolates from peach.

DISCUSSION

The phytopathogenic bacterium *P. syringae* is becoming a quite widespread pathogen of fruit trees in Serbia, causing significant economic losses. Up to now, it was experimentally confirmed as a pathogen on pear, apple, peach, apricot, cherry, sour cherry, and plum trees, as well as raspberry plants (Gavrilović, 2004). Our research strategy was based on the finding that *Pseudomonas syringae* is associated with a number of strains parasitic on stone fruit trees in Serbia. In recent years, *P. syringae* has caused blossom blast, as well as necrosis of branches and trunks of pear, resulting in the death of whole pear trees. Some varieties of sour cherry (Reksele, Hayman's Rubin, Keleris) are affected by spottedness, leading to necrosis and shedding of 60-70% of the fruits. Blossom and shoot blight of raspberry caused by *P. syringae* is also becoming a widespread disease in Serbian regions such as Ivanjica and Arilje, where raspberry is cultivated commercially. New symptoms observed in Serbia (Gavrilović et al., 2009) include the sudden dieback of buds in peach trees, wilting of young leaves and flowers, and necrosis of wooden tissue around the bud base, followed by cancer formation.

The isolates investigated here showed homogeneity in Gram staining, oxidative metabolism of glucose, and LOPAT tests (Table 1). These characteristics confirmed that the pathogen is *Pseudomonas syringae* (Braun-Kiewnick and Sands, 2001; Gavrilović, 2004).

With respect to their pathogenic characteristics, the investigated strains belong to two distinct groups. The first group contains varieties from peach, pear, apple, and raspberry, exhibiting typical characteristics of *P. s.* pv. *syringae*. The other group comprises isolates from cherry and plum buds and sour cherry fruits, showing characteristics typical of pv. *morsprunorum*. Our findings are in accordance with previous research on characteristics of varieties (Arsenijević, 1997; Gavrilović, 2004; Gavrilović and Ivanović, 2008).

Three groups are differentiated on the basis of their biochemical characteristics using GATT tests. The first group includes isolates from the peach, apple, and pear. They hydrolyze gelatin and esculin, but do not form thyrosinase or metabolize tartrate, proving that they are *P. s.* pv. *syringae*. The second group comprises strains from cherry and plum, with a negative reaction in the gelatin and esculin tests, but forming thyrosinase and using tartrate in their metabolic processes, indicating that they are *P. s.* pv. *morsprunorum*. Our results confirm previous data (Burkowitz and Rudolph, 1994; Gavrilović, 2004).

The isolates from sour cherry and raspberry comprise a third group, i.e., isolates that exhibit characteristics of both previously described groups, which is in agreement with previous findings (Sobiczevski, 1984; Balaž and Arsenijević, 1989; Gavrilović, 2004). The presence of intermediate varieties indicates that in addition to biochemical characterization, molecular methods are necessary for isolate identification.

Detection of differences among *Pseudomonas syringae* strains was successfully performed using the BOX-PCR method. This kind of characterization was here used for the first time to discriminate *Pseudomonas syringae* isolates originating from fruit trees in Serbia. The observed disparity at the level of genetic diversity is consistent with previous studies (Scortichini et al., 2003; Natalini et al., 2006).

Genetic fingerprints were determined for strains of *Pseudomonas syringae* isolated from peach, pear, apple, plum, sour cherry, and raspberry. The different BOX-PCR fingerprints detected among strains isolated from different hosts show that various strains of *Pseudomonas syringae* cause disease on the different plants. However, each strain is specialized to provoke unique symptoms on fruit trees, this specialization being correlated with the distribution of BOX sequences. The relationship with a particular host appears to affect the distribution of repetitive sequences, resulting in fingerprints unique to specific strains.

Diversity of the obtained *Pseudomonas syringae* isolates is further indicated by the different inhibitory activity of *Bacillus* sp. strains.

Based on the results obtained in this study, we are able to conclude that the population of the bacterium *P. syringae* from fruit trees in Serbia is heterogeneous with respect to genetic composition. It is interesting to note that closely related bacteria with similiar biochemical characteristics can have divergent rep-PCR profiles.

Our experiments demonstrate the potential of rep-PCR fingerprinting as a strong diagnostic tool in establishing differences among *Pseudomonas syringae* strains of various origin. It is of utmost importance to establish the taxonomic status of strains of *P. syringae* originating from diseased fruits of sour cherry, as well as from blighted blossoms, shoots, and flower clusters of raspberry. This subject is of scientific and practical significance as a means of establishing the host range of strains and elaborating new directions in studying the epidemiology of these pathogens.

Our further strategy in investigation of *P. syringae* strains from fruit trees in Serbia will involve the use of different primer sets, leading to specific conclusions about diversity or similarity among strains and pathovars. Moreover, such a strategy will help us to establish the similarity or diversity of *P. syringae* strains isolated from field crops, which are also known as bacterial hosts.

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ПРОУЧАВАЊЕ РАЗНОВРСНОСТИ БАКТЕРИЈЕ *PSEUDOMONAS SYRINGAE* ПОРЕКЛОМ СА РАЗЛИЧИТИХ ВОЋАКА У СРБИЈИ

Ж. ИВАНОВИЋ[1], СВЕТЛАНА ЖИВКОВИЋ[1], МИРА СТАРОВИЋ[1], ДРАГАНА ЈОШИЋ[2], С. СТАНКОВИЋ[3] и В. ГАВРИЛОВИЋ[1]

[1]*Институт за зашиту биља и животну средину,* 11000 Београд, Србија
[2]*Институт за земљиште,* 11000 Београд, Србија
[3]*Биолошки факултет, Универзитет у Београду,* 11000 Београд, Србија

Pseudomonas syringae је широко распрострањена и економски значајна фитопатогена бактерија, са широким кругом домаћина који укључује воћке, ратарске, повртарске и украсне биљке. *Pseudomonas syringae* у Србији је експериментално потврђен као паразит крушке, јабуке, кајсије, бресквe, трешње, вишње, шљиве и малине. Циљ ове студије био је да утврди постојање евентуалних разлика између сојева изолованих са различитих врста воћака у Србији. Проучаване су патогене и биохемијске особине сојева. BOX-PCR је коришћен за добијање профила изолата *Pseudomonas syringae* у циљу идентификације сојева који се не могу утврдити другим методама. Различити сојеви рода *Bacillus* су тестирани у циљу утврђивања њихове *in vitro* инхибиторне активности. Сојеви рода *Bacillus* су показали инхибиторну активност само на *P. syringae* изолованих са бресквe. Добијени резултати показали су да је популација бактерије *Pseudomonas syringae* пореклом са воћа у Србији врло разноврсна.