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Original scientific paper

GENETIC CHARACTERIZATION OF PATHOGENIC FLUORESCENT PSEUDOMONADS ISOLATED FROM NECROTIC CHERRY AND PLUM BUDS IN SERBIA

Veljko GAVRILOVIĆ[1], Žarko IVANOVIĆ[1], Tatjana POPOVIĆ[1], Svetlana ŽIVKOVIĆ[1], Slaviša STANKOVIĆ[2], Tanja BERIĆ[2] and Đorđe FIRA[2*]

[1]Institute for Plant Protection and the Environment, Belgrade, Serbia
[2]Faculty of Biology, University of Belgrade, Belgrade, Serbia

Gavrilović V., Ž. Ivanović, T. Popović, S. Živković, S.Stanković, T.Berić and Đ. Fira (2013): *Genetic characterization of pathogenic fluorescent pseudomonads isolated from necrotic cherry and plum buds in Serbia*. Genetika, Vol 45, No. 3, 953-961.

During past few years a symptoms of plum and cherry bud necrosis were observed in some regions with significant cherry production in Serbia. Gram negative, fluorescent, oxidative bacterial strains were isolated from the margin of necrotic tissue. All investigated strains are levan and HR positive, while negative results are recorded in oxidase, pectinase and arginin dihydrolase tests (LOPAT+- - - +). Symptoms similar to those observed in natural infection were obtained after artificial inoculation of cherry leaf scares and dormant one year old cherry shoots. Investigated strains as well as reference strain of *P. syringae* pv. *morsprunorum* cause the superficial necrosis on artificially inoculated immature cherry fruits, but negative results were recorded in immature pear and lemon fruit tests as well as syringae leaves and bean pods. Gelatin and aesculin tests were negative and tyrosinase and tartrate were positive. Investigated strains isolated from necrotic cherry buds had identical REP-PCR pattern with reference strain of *P. syringae* pv. *morsprunorum*. On the basis of obtained results, it was concluded that this bacterium is causal agent of cherry trees bud necrosis in Serbia.

Key words: bud necrosis, cherry, GATT, pathogenicity, *P. syringae* pv. *morsprunorum*, plum, REP-PCR

INTRODUCTION

Pseudomonas syringae is one of the most important bacterial plant pathogens, as the causative agent of diseases of fruit trees. Pathovars of *P. Syringae* are one of major problems in

Corresponding author: Đorđe Fira, Faculty of Biology, University of Belgrade, Belgrade, Serbia,Tel/fax: +381 11 2639882, E-mail: fira@bio.bg.ac.rs

fruit-producing regions, because of significant economic losses and lack of successful strategies for their control (KENNELLY *et al.,* 2007). They can infect numerous hosts, including stone fruits, pome fruits and other trees, as well as grasses and crop plants. Most of the pathovars of *P. syringae* have relatively narrow host range, but some of them, like strains of *P. syringae* pv. *syringae*, are pathogenic for many different plant species (BRADBURY, 1986, IVANOVIĆ *et al.,* 2012). Among diseases of stone fruit trees, bacterial canker, caused by strains of *P. syringae* pv. *syringae and* pv. *morsprunorum* are very important, because of great potential losses in orchards. The symptoms of this disease are visible on leafs and fruit as brown spots, followed by the blossom blast, spur dieback and cankers which are associated with gummy ooze from woody tissue and death of branches and entire trees.

During the past few years, severe necrosis of sweet cherry and plum leaf and flower buds was observed in the regions of Šabac, Topola, and Belgrade. At the beginning of vegetative season, diseased cherry and plum buds failed to develop leaves and flowers. Tissue around the buds was dark colored and expressed as ellipsoid, sunken lesions. After the removal of superficial epidermal layer, dark brown tissue necrosis was observed. Based on the type of symptoms, it was assumed that the causal agent of this disease is phytopathogenic bacterium *Pseudomonas syringae*.

Therefore, the aim of this study was to identify and characterize the causal agent of this disease.

MATERIALS AND METHODS

Pathogen isolation was conducted according to standard procedures for pseudomonads using sucrose nutrient agar (SNA) (nutrient agar, containing 1.5 % peptone, 0.3 % meatextract, 0.5 % NaCl, 0.03 % K_2HPO_4, 1.8 % agar and supplemented with 0.5 % sucrose) and King's B medium (KB) (2 % peptone, 1.5 % K_2HPO_4, 1.5 % $MgSO_4 \times 7H_20$, 1 % glycerol, 1.5 % agar). Single cell colonies were transferred on to nutrient agar amended with 2% glycerol (NGA) slants and stored at 4°C for further studies. Five strains from sweet cherry and five strains from plum trees from several production localities in Serbia (Belgrade, Šabac, Valjevo) were chosen for further study (Tab. 1). The strains were compared with the reference strains of *Pseudomonas syringae* pv. *syringae, Pseudomonas syringae* pv. *morsprunorum* and *Pseudomonas syringae* pv. *avii* –recently reported as a *P. syringae* pathovar (MENARD *et al.*, 2003) affecting wild cherry (only for REP PCR analysis). Reference strains were obtained from Collection Française des Bactéries Phytopathogènes, INRA, Angers, France (Tab. 1).

Pathogenicity of the strains was tested on sweet cherry and plum leaf scars, made by removal of leaves. The scars were inoculated by pipetting a drop of bacterial suspension (adjusted to aproximately 10^8 cfu/ml). Sweet cherry dormant shoots (cv. Cordia) as well as plum shoots (cv. Stenley) was used in pathogenicity tests. Pathogenicity of the strains was also investigated by inoculation of sweet cherry (cv. Burlat), pear (cv. Villiams), and lemon fruits, lilac leaves and bean pods (BURKOVITZ and RUDOLPH, 1994; GAVRILOVIĆ, 2006; BULTREYS and KALUZNA, 2010).

***Identification of strains*.** The biochemical tests important for the differentiation of the *P. syringae* pathovars (LOPAT and GATT) were also studied (BROWN-KIERNICK and SANDS, 2001) as well as Gram stain and standard biochemical tests for bacterial identification:

hydrolysis of gelatin, aesculin and starch; tyrosinase activity; growth on Tween 80; DL-lactate; urease and catalase tests, growth at 4°C and 41°C; nitrate reduction; growth at 3% and 5% NaCl and utilization of different sugars.

Table 1. Pseudomonas syringae strains

Strain	Isolate	Host	Origin
P. syringae pv. *morsprunorum*	C1	Sweet cherry	This work
P. syringae pv. *morsprunorum*	C2	Sweet cherry	This work
P. syringae pv. *morsprunorum*	C3	Sweet cherry	This work
P. syringae pv. *morsprunorum*	C4	Sweet cherry	This work
P. syringae pv. *morsprunorum*	C5	Sweet cherry	This work
P. syringae pv. *morsprunorum*	P1	Plum	This work
P. syringae pv. *morsprunorum*	P2	Plum	This work
P. syringae pv. *morsprunorum*	P3	Plum	This work
P. syringae pv. *morsprunorum*	P4	Plum	This work
P. syringae pv. *morsprunorum*	P5	Plum	This work
P. syringae pv. *morsprunorum*	CFBP2119	Sweet cherry	Reference strain*
P. syringae pv. *syringae*	CFBP11	Pear	Reference strain*
P. syringae pv. *avii*	CFBP3846	Pear	Reference strain*

* From a Collection Française des Bactéries Phytopathogènes, INRA, Angers, France

Isolation of DNA and REP PCR

Total genomic DNA was prepared as follows. Cultures were grown on NAS (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.

Amplification was performed in a total volume of 25 µl containing 67 mM Tris-HCl (pH 8.8); 25 mM MgCl2; 125 µM of dATP, dCTP, dGTP, and dTTP each; 2 units of Taq DNA polymerase (Fermentas, Lithuania); and 100 pmol of REP1R-I and REP2-I primer. A 40-ng quantity of genomic DNA or distilled water as a negative control was added to the reaction tubes. The primers were a sequences corresponding to REP, a subunit of the REP element (LUPSKI *et al.*, 1992): (REP1R-I [5'-IIIICGICGICATCIGGC-3'] and REP2-I [5'-ICGICTTATCIGGCCTAC-3']). The PCR protocols with REP primer are referred to as REP-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; 35 cycles of denaturation at 94°C for 1 min; annealing at 40°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.

RESULTS AND DISCUSSION

Ten pathogenic strains of *P. syringae* were isolated from necrotic tissue of plum and sweet cherry trees. Typical levan type colonies were developed on SNA and green fluorescent colonies on KB medium after 2 days at 26°C**.** Isolated strains were gram negative with oxidative metabolism of glucose and showed the LOPAT characteristics of the *Pseudomonas syringae* group I (Tab. 2), being positive for levan production, expressed ability to produce a hypersensitivity reaction in tobacco, they were oxidase and arginine dihydrolase negative, and lacking pectolytic activity.

Table 2. Bacteriological properties of the Pseudomonas syringae reference strains and isolates from sweet cherry and plum

Test / Strain	**C1**	**C2**	**C3**	**C4**	**C5**	**P1**	**P2**	**P3**	**P4**	**P5**	**RS1**	**RS2**
Gram reaction	-	-	-	-	-	-	-	-	-	-	-	-
O/F test	O	O	O	O	O	O	O	O	O	O	O	O
Levan	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Potato soft rot	-	-	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-
Tobacco HR	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of:												
Gelatine	-	-	-	-	-	-	-	-	-	-	-	+
Aesculine	-	-	-	-	-	-	-	-	-	-	-	+
Tyrosinase activity	+	+	+	+	+	+	+	+	+	+	+	-
Tartarate	+	+	+	+	+	+	+	+	+	+	+	-
Starch	-	-	-	-	-	-	-	-	-	-	-	-
Tween 80	-	-	-	-	-	-	-	-	-	-	-	-
DL-lactate	-	-	-	-	-	-	-	-	-	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 41°C	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
Utilization of:												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-
Erythritol	+	+	+	+	+	+	+	+	+	+	+	+

RS1: CFBP2119; RS2CFBP11

After inoculation test, ellipsoid, sunken, necrotic lesions, slightly smaller than in natural infection, were observed around the inoculation spot on sweet cherry shoots cv. Cordia and on plum (cv. Stenley), also. Green fluorescent, oxidase negative bacterial strains with the ability to produce levan and a hypersensitivity reaction in tobacco were recovered from the inoculated symptomatic tissue, thus confirming the Koch's postulates.

The investigated strains, as well as the reference strain CFBP 2119 of *P. syringae* pv. *morsprunorum* induced superficial lesions on the immature sweet cherry fruits, whereas inoculation tests on pear, and lemon fruits, lilac leaves and bean pods were negative. On the other hand, *P. syringae* pv. *syringae* CFBP 11 reference strain reacted positively in all inoculation tests. However, this strain induced severe symptoms on the immature sweet cherry fruits compared to the investigated strains expressed as sunken necrotic lesions. These results were in compliance with the results of the pathogenicity tests of other authors for *Pseudomonas syringae* bacterium (BURKOWITZ and RUDOLPH, 1994; GAVRILOVIĆ, 2006; GILBERT *et al.*, 2009; KALUZNA and SOBICZEWSKI, 2009; BULTREYS and KALUZNA, 2010; KALUZNA *et al.* 2010a).

Based on the results of our study we concluded that the investigated strains had the same pathogenic characteristics as *P. syringae* pv. *morsprunorum*. These investigations confirmed the importance of pathogenicity tests in the differentiation of *P. syringae* pathovars (pv. *syringae* and pv. *morsprunorum*) previously implied by other authors (BURKOWITZ and RUDOLPH, 1994; GAVRILOVIĆ, 2006; KALUZNA and SOBICZEWSKI, 2009; KALUZNA *et al.*, 2010a). Our results also verified previous reports on the role of leaf scars as one of the *P. syringae* pv. *morsprunorum* entry points (HINRICH- BERGER, 2004).

The biochemical tests important for the differentiation of the *P. syringae* pathovars were also studied (BROWN-KIERNICK and SANDS, 2001). The results of these tests are given in Tab. 2. Based on these data, the strains isolated from necrotic sweet cherry an plum buds had uniform biochemical characteristics typical for *P. syringae* pv. *morsprunorum* (BURKOWITZ and RUDOLPH, 1994; YOUNG and TRIGGS, 1994; BROWN-KIERNICK, 2001; KARIMI-KURDISTANI and HARIGHI, 2008; KALUZNA *et al*., 2010b). On the other hand, the investigated strains differed from the *P. syringae* pv. *syringae* reference strains in the results of the gelatine and esculine hydrolyses tirosinase activity and metabolism of L-tartrate (GATT) as well as DL-lactate metabolism (Tab.2). In addition, the investigated strains did not produce syringomycin in the test with *Geotrichum candidum* (GROSS and DE VAY, 1977) (data not shown) whereas coronatine production induced by some *P. syringae* pv. *morsprunorum* strains (KENELLY *et al*., 2007) will be further investigated.

PCR fingerprinting using primers corresponding to repetitive (REP) was investigated as a method to distinguish the pathovars of *Pseudomonas syringae.* After amplification of total DNA with the REP- followed by agarose gel electrophoresis, the tested five isolates originated from cherry as well as other five strains from plum showed specific patterns of PCR products identical with the referent strain *Pseudomonas syringae* pv *morsprunorum* (Fig. 1). The size of the amplification products ranged from 100 bp up to 6000 bp. The fingerprint patterns of the strains were highly reproducible with the REP primer set. The results presented here demonstrated the usefulness of the PCR method with the REP- primers for genetic characterization and identification of *P. syringae* pathovars. REP-PCR as a simple, quick and inexpensive diagnostic method is particularly useful for routine diagnostic analyses. Our results suggest that this is the case, isolates of *P. syringae* pv. *morsprunorum* were isolated from cherry and plum buds, obtained from various locations in the Serbia over the last several years (2006-

2010) have identical fingerprint profiles. Likewise, we have analyzed isolates of referent strain CFBP 2119 *P. syringae* pv. *morsprunorum* from the French collection of phytopathogenic bacteria, which have an identical profile to the isolates from Serbia. Comparison of the genomic fingerprint profiles of different isolates within a pathovar, separated by time or distance, supports the notion that the profiles remain stable. The fingerprint patterns of a strain were highly reproducible with tested primer set. Thus, the PCR technique REP- primers is a rapid, simple, reproducible, and low cost method to identify strains of the *P. syringae* pv. *morsprunorum*. Reliability of this method in detection and differentiation of *P. syringae* pathovars is also proved by other researchers (VICENTE and ROBERTS, 2007; KALUZNA *et al*., 2010a; KALUZNA *et al*, 2010b).

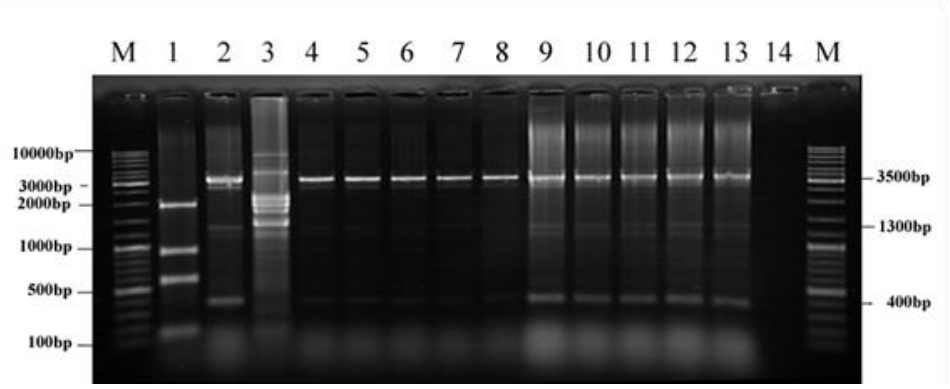

Fig. 1. REP-PCR fingerprint patterns obtained from *P. syringae* reference strains and isolates from sweet cherry and plum. Lanes: **M**, DNA molecular size marker (GeneRulerTM DNA Ladder Mix); **1**. *P. syringae* pv. *syringae* CFBP 11; **2**. *P. syringae* pv. *morsprunorum* CFBP 2119; **3**. *P. syringae* pv. *avii* CFBP 3846; **4**. *P. syringae* pv. *morsprunorum* C1; **5**. *P. syringae* pv. *morsprunorum* C2; **6**. *P. syringae* pv. *morsprunorum* C3; **7**. *P. syringae* pv. *morsprunorum* C4; **8**. *P. syringae* pv. *morsprunorum* C5; **9**. *P. syringae* pv. *morsprunorum* P1; **10**. *P. syringae* pv. *morsprunorum* P2; **11**. *P. syringae* pv. *morsprunorum* P3; **12**. *P. syringae* pv. *morsprunorum* P4; **13**. *P. syringae* pv. *morsprunorum* P5; **14**. control sample without DNA.

The results of our study revealed that *P. syringae* became widely distributed and very destructive pathogen of fruit trees in Serbia. Up-to-date, it was reported to be most dangerous as a pathogen of pear, apricot and plum trees. However, the strains isolated from these fruit trees belonged to *P. syringae* pv. *syringae* according to their pathogenic and biochemical features (GAVRILOVIĆ, 2006; GAVRILOVIĆ *et al.,* 2008). BOX PCR analyses of Serbian strains showed high heterogeinity dependent on the host (IVANOVIĆ *et. al*, 2009). Heterogeinity of *P. syringae* pv. *syringae* population have also been reported by several authors (NATALINI *et al.*, 2006). On the other hand, high similarity of the investigated strains isolated from sweet cherry in REP-PCR, could be attributed to the closeness of the localities from which the strains were isolated. Further investigations of *P. syringae* as a sweet cherry pathogen are needed in order to determine

whether pathovar *syringae* is present in sweet cherry trees in Serbia, particularly because this bacterium is distributed worldwide causing great production losses (KENELLY *et al*., 2007; BULTREYS and KALUZNA, 2010). Moreover, this implies the necessity for studies of the structure and seasonal dynamics of *P. syringae* epiphytic population (RENICK *et al*., 2008) which plays an important role in pathogen epidemiology.

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GENETIČKA KARAKTERIZACIJA PATOGENIH FLUEROSCENTNIH PSEUDOMONADA IZOLOVANIH IZ NEKROTIČNIH TREŠNJI I ŠLJIVA U SRBIJI

Veljko GAVRILOVIĆ[1], Žarko IVANOVIĆ[1], Tatjana POPOVIĆ[1], Svetlana ŽIVKOVIĆ[1], Slaviša STANKOVIĆ[2], Tanja BERIĆ[2] i Đorđe FIRA[2*]

[1]Institut za zaštitu biljaka i spoljašnju sredinu, Beograd, Srbija
[2]Biološki fakultet, Univerzitet Beograde Beograd, Srbija

Izvod

Tokom nekoliko poslednjih godina u nekim regionima Srbije, značajnim za proizvodnju trešanja, uočeni su simptomi nekroze pupoljaka ovog voća. Sa margina nekrotičnog tkiva izolovani su sojevi gram negativnih, fluorescentnih, oksidativnih bakterija. Svi ispitivani sojevi su bili levan i HR pozitivni dok su negativni rezultati zabeleženi u oksidaza, pektinaza i dihidrolaza testovima (LOPAT +---+). Simptomi slični onima zabeleženim u prirodnim infekcijama dobijeni su nakon veštačke inokulacije lisnih ožiljaka i dormantnih jednogodišnjih sadnica trešnje. Ispitivani sojevi, kao i referentni soj *Pseudomonas syringae* pv. *morsprunorum* izazivaju površinsku nekrozu na veštačkim nezrelim plodovima trešnje, ali negativni rezultati su zabeleženi na nezrelim plodovima kruške i limuna kao i na lišću jorgovana i mahunama pasulja. Ispitivani sojevi izolovani sa nekrotičnih pupoljaka trešnje i šljive imali su identičan REP-PCR profil traka kao i referentni soj *P. syringae* pv. *morsprunorum*. Dobijeni rezultati ukazuju na to da je uzročnik nekroze pupoljaka trešnje bakterijska infekcija sojem *P. syringae* pv. *morsprunorum*.

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