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Scientific paper

Pseudomonas syringae – Pathogen of Sweet Cherry in Serbia

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SUMMARY

Characteristics of pathogenic *Pseudomonas* bacterial strains isolated from cherry in Serbia are presented in the article. Two types of symptoms were observed on cherry trees at few localities with intensive production in Serbia (Belgrade, Čačak, Topola, Šabac, Novi Sad). The first symptom is bud necrosis and the second bacterial canker of cherry branch.

Gram negative, fluorescent, oxidative bacterial strains were isolated from the margin of necrotic tissue. All investigated strains were levan and HR positive, while negative results were recorded for oxidase, pectinase and arginin dihydrolase tests (LOPAT+- - - +).

Based on pathogenicity tests and differential GATT tests, investigated strains were divided in two distinct groups: the first group consisted of strains isolated from necrotic cherry branch which caused necrosis on artificially inoculated cherry, pear and lemon fruits, syringae leaves and bean pods, were gelatin and aesculin positive, and tyrosinase and tartrate negative (typical characteristics of *P.s.* pv. *syringae*). Contrary, second group strains were isolated from necrotic cherry buds, showed negative results in mentioned pathogenicity tests, gelatin and aesculin tests were negative, while tyrosinase and tartrate were positive (typical characteristics of *P.s.* pv. *morsprunorum*).

REP PCR analyses showed that strains isolated from necrotic cherry buds belong to *P. s* pv. *morsprunorum* compared to referent strain**.** In contrast, isolates obtained from necrotic cherry branches had unique fingerprint profiles but different from all reference strains.

According to the obtained results it was concluded that both pathovars of *P. syringae* (*syringae* and *morsprunorum*) cause necrosis of cherry trees in Serbia.

Keywords: Cherries, *Pseudomonas syringae;* Pathogenicity; GATT tests, REP PCR

INTRODUCTION

Production of sweet cherry in Serbia has greatly expanded in the last decade, reaching the annual level of over 27,000 tons, which is several times higher than earlier (Sredojević, 2011). Selection of new, less vigorous rootstocks significantly increased the number of less luxuriant cherry trees per unit area, making the organization of picking much easier. Also, growing of new, highly productive cultivars has provided high yields and quality fruits which are easily sold at both, domestic and foreign markets. These are some of the major reasons for intensifying the production of sweet cherry fruits in our country.

All the above mentioned resulted in significant expanding of sweet cherry growing region in Serbia and, besides well-known areas by the river Danube near Belgrade, sweet cherry is now intensively grown in Vojvodina, western and southern Serbia, Šumadija and other regions.

In recent years, however, the symptoms of bud necrosis and branch drying have been observed in these new orchards in which contemporary agro-technical measures were applied. Dark purple, elliptic spots, 2-3 cm long with abundant gum production, were formed around necrotic buds. Within the spots the tissue subsided and was clearly different from adjacent, healthy tissue. Removing of the superficial layer revealed brown colored tissue necrosis. The other type of symptoms was decay of several-year old branches. The first signs of the decay could be observed in early spring, when leaves became wilted and turned brown, staying attached to diseased branches. Tissue necrosis could be seen on these branches, with canker at borderline area between diseased and healthy tissue. Removing of the superficial layer also revealed tissue necrosis of damp consistence. In both cases, the process of necrosis was followed by abundant production of gum.

Since the observed symptoms are characteristic for *Pseudomonas syringae* infections, the investigation was carried out to determine if this bacterium caused necrosis of sweet cherry branches and buds, and to perform detailed characterization of the obtained strains.

MATERIAL AND METHODS

Samples from diseased sweet cherries with symptoms of bud and branch necrosis were collected at the localities of Belgrade, Šabac, Topola and Novi Sad, in the period 2004-2010. Samples were collected in early spring, immediately after characteristic symptoms had been observed (Table 1).

Table1. List of investigated strains

Isolate	Symptom	Locality	Year of isolation
IZB-101	Branch necrosis	Topola	2009
IZB-102	"	"	"
IZB-103	"	"	"
IZB-104	"	Šabac	2010
IZB-105	"	"	"
IZB-109	"	Novi Sad	"
IZB-110	"	"	"
IZB-90	Bud necrosis	Belgrade	2004
IZB-91	"	"	"
IZB-92	"	Šabac	"
IZB-93	"	"	"
IZB-94	"	"	"
IZB-95	"	"	"
IZB-96	"	"	"
IZB-97	"	"	"
IZB-98	"	"	"
IZB-99	"	"	"

Isolation and pathogenicity testing

Isolation of the bacterium was performed using standard method of smearing onto culture media, after macerate was made by soaking the borderline fragments in a sterilized ceramic mortar with 1 ml of phosphate buffer. The isolation was carried out on the NA enriched with 5% sucrose (SNA), and King's B medium (Klement, 1990; Braun-Kiewnick and Sands, 2001).

Pathogenicity of the obtained isolates was tested by infiltration of the bacterial suspension (10^8 cfu/ml) in tobacco and pelargonium leaves (HR), and by inoculation of unripe pear, sweet cherry and lemon fruits as well as bean pods using medicinal syringae. Lilac leaves were inoculated by dipping of leaf petal in bacterial suspension (10^8 cfu/ml) (Klement, 1990; Arsenijević, 1997; Gavrilović, 2006).

Biochemical and physiological characteristics

Biochemical and physiological characteristics essential for the identification of *Pseudomonas syringae* were examined and differential tests for its pathogenic varieties *syringae* and *morsprunorum* were carried out: formation of levan and activity of oxidase, pectolytic enzymes and arginine dehydrolase (LOPAT test), as well as gelatin and esculin hydrolysis, tyrosinase production and metabolism of tartrates (GATT test). Gram's

method of differentiation and glucose metabolism test (O/F), were also conducted (Lelliott et al., 1966; Lelliott and Stead, 1987; Sands, 1990; Burkowicz and Rudolph, 1994; Arsenijević, 1997; Brown-Kiewnick and Sands, 2001; Gavrilović, 2006).

REP-PCR

In this study the usefulness of PCR method with REP, primers for genetic characterization and identification of *P. syringae* pathovars was demonstrated.

Total genomic DNA was prepared using a modification of the procedure given by Ausubel et al. (1992). Cultures were grown on NAS (sucrose nutrient agar) medium for 48h 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 1h. 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 was added to the reaction tubes as a negative control. The primers were sequences corresponding to REP, a subunit of the REP element (Lupski and Weinstock al., 1992): (REP1R-I [5'-IIIICGICGICATCIGGC-3'] and REP2-I [5'-ICGICTTATCIGGCCTAC-3']). The PCR conditions were as previously described (de Bruijn, 1992). 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 2h at 5 V/cm, stained with ethidium bromide, and visualized under UV illumination. Fingerprints generated from different strains were compared visually.

In this investigation, the following strains were used as check strains: CFBP 11 (*P. syringae* pv. *syringae*), CFBP 2119 (*P. syringae* pv. *morsprunorum,* race 1) and CFBP 3846 (*P. syringae* pv. *avii,* only for PCR analylsis). These strains originated from French collection of phytopathogenic bacteria (Angers).

RESULTS

Although samples of diseased cherries from different localities were collected during the whole vegetation period, successful isolations of the bacteria were performed immediately after the appearance of the first symptoms, in early spring (April and May).

On the NA medium enriched with sucrose (NAS) the bacteria formed greyish-white colonies of the levan type, 2-3 mm in diameter, which were shiny, smooth and mucous. They could be seen after 2-3 days of growth at the temperature of 24-26 °C . On the King's B medium, formed colonies were white, 2 mm in diameter and fluorescent under UV light.

Pathogenicity and biochemical characterization

All the examined isolates caused HR in tobacco and pelargonium, however based on other pathogenic characteristics, two groups of strains were differentiated (Table 2).

The group of strains isolated from necrotic several-year old sweet cherry branches caused subsided brown-black spots on the inoculated fruits of sweet cherry (cv. Burlat), pear (cv. Williams) and lemon. This group of strains also caused brown spots with characteristic reddish-orange halo on inoculated bean pods, as well as the necrosis of lilac leaf petal and veins and mesophyl tissue.

The strains isolated from necrotic sweet cherry buds caused superficial brown spots on inoculated sweet cherry fruits, and light brown spots on inoculated bean pods significantly differed from those caused by the first group of strains, which was characterized as negative result. Negative results were recorded in inoculation tests on unripe pear and lemon fruits as well as lilac leaves. Such results were also obtained with the check strain *P. s.* pv. *morsprunorum* CFBP 2119.

Table 2. Pathogenicity of ivestigated strains

Test	*IZB 101-110	**IZB 92-99	CFBP 11	CFBP 2119
HR (tobacco, pelargonium)	+	+	+	+
Immature fruits of:				
pear	+	-	+	-
cherry	+	+/-	+	+/-
lemon	+	-	+	-
syringae leaves	+	-	+	-
bean pods	+	-	+	-

+ positive results
- negative results
+/- superficial spots on inoculated fruits

* Strains isolated from necrotic cherry branch
** Strain isolated from necrotic cherry buds
CFBP 11 *P.s.* pv. *syringae* check strain
CFBP 2119 *P. s.* pv. *morsprunorum* check strain

Table 3. Bacteriological characteristics of investigated strains

Test	*IZB 101-110	**IZB 92-99	CFBP 11	CFBP 2119
Gram	-	-	-	-
O-F test	O	O	O	O
Fluorescence	+	+	+	+
Levan	+	+	+	+
Oxidase	-	-	+	-
Pectinase	-	-	-	-
Arginin dehidrolase	-	-	-	-
Gelatin (G)	+	-	+	-
Aesculin (A)	+	-	+	-
Tyrosinase (T)	-	+	-	+
Tartrate (T)	-	+	-	+

* Strains isolated from necrotic cherry branch
** Strain sisolted from necrotic cherry buds
CFBP *P.s.* pv. *syringae* check strain
CFBP 2119 *P. s. pv morsprunorum* check strain

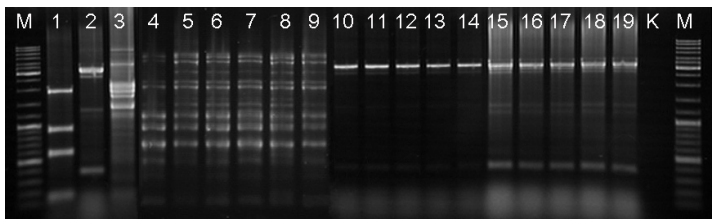

Figure 1. Agarose gel electrophoresis of as repetitive-sequence – based polymerase chain reaction (REP-PCR) fingerprint patterns obtained from *P. syringae.* Lane M show the DNA molecular size marker (GeneRulerTM DNA Ladder Mix), CFBP-11 (lanes 1), CFBP-2119 (lanes 2), CFBP-3846 (lanes 3), *P. syringae* isolate from cherry (lane 4-19), and negative control (lane K)

CFBP 11 (*P. syringae* pv. *syringae*)
CFBP 2119 (*P. syringae* pv. *morsprunorum*)
CFBP 3846 (*P. syringae* pv. *avii*)

All the investigated isolates were gram-negative, metabolized glucose only in aerobic conditions, created fluorescent pigment on the King's B medium; they formed levan and the result of testing the production of oxidase, pectolytic enzymes and arginine dehydrolase was negative. According to the results of differential tests for pathogenic varieties *syringae* and *morsprunorum*, the investigated strains formed two, clearly distinct groups. The first group consisted of strains obtained from necrotic sweet cherry branches, which hydrolyzed gelatin and esculin, did not produce tyrosinase and did not metabolize tartrates. Strains of the second group originated from necrotic sweet cherry buds, did not hydrolyze gelatin and esculin, but reacted positively when tested for tyrosinase production and metabolism of tartrates (Table 3).

From the results of pathogenicity tests and bacteriological characteristics of the investigated isolates, it can be concluded that the isolates obtained from necrotic sweet cherry tissues belong to the pathogenic varieties *syringae* and *morsprunorum* of the bacterium *P. syringae.*

REP-PCR analysis

PCR fingerprinting using primers corresponding to repetitive (REP) was investigated as a method to distinguish pathovars of *Pseudomonas syringae.* After amplification of total DNA with the REP- followed by agarose gel electrophoresis, the tested isolates originated from cherry showed specific patterns of PCR products identical with the reference strain *Pseudomonas syringae* pv *morsprunorum.* The size of the amplification products ranged from 100 bp to 6000 bp. The fingerprint patterns of the strains were highly reproducible with the REP primer set.

Two distinct REP-PCR fingerprints were generated for *P. syringae* isolates collected in Serbia (Figure 1). These results indicated that *P. syringae* is present on cherry in Serbia, with two distinct genotypes. Strains IZB 90-99, isolated from necrotic cherry buds had identical REP-PCR pattern with reference strain CFBP 2119, and could be identified as *P. syringae* pv. *morsprunorum.* In contrast, isolates IZB 101-106 obtained from necrotic cherry branch had unique fingerprint profiles but different from all reference strains. Together, these results indicate that *P. syringae* parasites cherry with two heterogeneous pathovars.

DISCUSSION

Phytopathogenic bacterium *P. syringae* is a commercially important pathogen of stone-fruit, wide-spread all over the world (Little et al., 1998; Hinrichs-Berger, 2004b; Kenelly et al., 2007; Renick et al., 2008). Besides stone-fruit, it can also cause great damages to pome fruits, especially to pear (Spotts and Cervantes, 1994; Natalini et al., 2006; Gavrilović et al., 2009).

In Serbia, *P. syringae* has been experimentally confirmed as the pathogen of pear, apricot, sour and sweet cherry, plum, peach and raspberry, causing the symptoms of blossom blast, branch, trunk and fruit necrosis, shoot blight and the necrosis of leaf and flower buds (Gavrilović et al., 2004a, 2008, 2009; Gavrilović, 2006, 2009). Its higher incidence in sweet cherry indicates its spreading and, taking into account a wide range of hosts, it can be assumed that it could threaten future successful production not only of stone, but also of pome fruit. In recent years, this bacterium has been observed as raspberry pathogen, causing shoot and blossom blight (Gavrilović et al., 2004b; Obradović et al., 2008).

Although the observed symptoms are characteristic of the infection with *P. syringae* (especially the type of necrosis and abundant production of gums), the final diagnosis requires isolation of the pathogen since some phytopathogenic fungi (*Botryosphaeria, Leucostoma*) cause similar symptoms on stone-fruits (Spotts et al., 1990; Jones and Sutton, 1996; Gavrilović, 2006).

The obtained results confirm the great importance of pathogenicity tests for differentiation of *P. syringae* varieties, which was also emphasized by many other authors (Gavrilović, 2006; Kaluzna and Sobiczewski, 2009; Bultreys and Kaluzna, 2010; Gilbert et al., 2010; Kaluzna et al., 2010a). On the basis of their pathogenic characteristics, the investigated strains were classified into two, clearly distinct groups: the first, consisted of HR positive strains isolated from necrotic buds, which caused superficial brown spots on inoculated sweet cherry fruits, but did not cause the necrosis on inoculated pear and lemon fruits, bean pods and lilac leaves, thus expressing the characteristics of pv. *morsprunorum*. The second group of strains reacted positively in all pathogenicity tests and expressed the characteristics of pv. *syringae*. Also, some of these tests could be important for detection of different virulence levels of *P. syringae* strains, which especially refers to the inoculation of lilac leaves (Gilbert et al., 2009).

Differential biochemical tests for pathogen varieties (GATT) also indicate the existence of two, clearly distinct groups of *P. syringae* strains, isolated from diseased sweet cherry tissues. The first group, isolated from necrotic buds, expressed characteristics of pv. *morsprunorum* and the second, isolated from necrotic branches expressed characteristics of pv. *syringae* (Table 3). Reliability of these tests in differentiation of pathogenic *P. syringae* varieties was previously confirmed (Latorre

and Jones, 1979; Burkowicz and Rudolph, 1994; Hinrichs-Berger, 2004b; Gavrilović, 2006; Kaluzna et al., 2010). However, among investigated *P. syringae* strains isolated from stone-fruits, some intermediary strains were detected, expressing the characteristics of both pathogen varieties in GATT tests (Sobiczewski, 1984; Balaž et al., 1988; Balaž and Arsenijević, 1989).

REP-PCR analysis also indicates that isolates from necrotic sweet cherry buds have typical characteristics of *P. s.* pv. *morsprunorum*. Moreover, they express the characteristics of race 1 of this bacterium, because in REP-PCR analysis they leave the identical "print" as the check strain of this bacterium CFBP 2119. This race consists of widely distributed and commercially harmful pathogen strains in sweet and sour cherry (Gilbert et al., 2009). Determination of these strains is very significant for epidemiology, as well as for the elaboration of control measures, which is mentioned below.

Contrary, strains isolated from necrotic cherry branches had unique fingerprint profiles but different from all reference strains. These results revealed that population of *P.s.* pv. *syringae* shows great genetic variability in REP-PCR analyses. This is in compliance with results of other authors, and primarily depends on localities of isolated strains (Scortichini et al., 2003; Natalini et al., 2006).

Reliability of REP-PCR analysis for determination and differentiation of *P. syringae* isolates was also confirmed by other authors (Vicente and Roberts, 2007; Gilbert et al., 2009; Kaluzna et al., 2010a, 2010b). In France, by this analysis, together with numerous other methods, a group of strains was isolated from a diseased tissue of wild sweet cherry, and these strains were later classified into a new pathogen variety *P.s.* pv. *avii* (Meńard et al., 2003).

PCR analysis, with different sets of primers (BOX and ERIC), also proved a great variety of *P. syringae* population originating from different host plants, and in some cases, among the strains from the same host plants and the same orchard (Little et al., 1998; Scortichini et al., 2003; Natalini et al., 2006). PCR analysis of *P. syringae* population in Serbia, using BOX primers, also revealed differences among strains which originated from different host plants (pear, sour cherry, plum, peach and raspberry) (Ivanović et al., 2009). Therefore, it can be assumed that strains from sweet cherry differ in some characteristics from strains originating from other hosts, which could be the subject of future investigations.

Sweet cherry is very susceptible host plant to the bacterium *P. syringae* all over the world. The infection of high intensity causes wide-spread necrosis of the branches. The necrosis can spread over fruits and, as a very common symptom, leaves become spotty followed by shot hole (Jones and Sutton, 1996; Kenelly et al., 2007; Renick et al., 2008). The bacterium could probably spread over tree-trunks, causing the whole trees to die off, since similar symptoms were recorded in apricot (Arsenijević, 1997), pear (Spotts and Cervantes, 1994; Gavrilović, 2009) and plum (Hinrichs-Berger, 2004b) trees. Although in this investigation two clearly distinct groups of strains were isolated from parts of the trees with two types of symptoms, we believe that pathogenic varieties of *P. syringae* should not be identified on the basis of symptoms, since both varieties can cause both types of symptoms.

P. syringae infects sweet cherry trees through leaf scars in the period of leaf falling (Freigoun and Crosse, 1975) when it is recommended to apply preparations with cooper to protect damaged tissue (Jones and Sutton, 1996). The infection of tree-trunks with *P. syringae* occurs after extremely low winter temperatures, and protection can be performed by wrapping special white bands round the trunks before the dormant period (Hinrichs-Berger, 2004a). Epiphytic population of the bacteria, which is present on leaves during vegetation, also plays an important role in epidemiology of *P. syringae*, as a sweet cherry pathogen (Latorre and Jones, 1979; Renick et al., 2008). The composition of this population and its seasonal dynamics on sweet cherry leaves in our agro-ecological conditions will be further investigated.

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Pseudomonas syringae – patogen trešnje u Srbiji

REZIME

U radu su prikazane patogene, bakteriološke odlike izolata bakterije *Pseudomnas syringae* poreklom iz trešnje, kao i njihova molekularna karakterizacija primenom REP-PCR metode. Dva tipa simptoma bolesti zapažena su u savremenim zasadima trešnje na području Beograda, Novog Sada, Topole i Šapca.

Prvi tip simptoma se ispoljava u vidu nekroze cvetnih i lisnih pupoljaka trešnje, a drugi u vidu nekroze višegodišnjih grana.

Iz nekrotičnog tkiva su izolovane Gram negativne bakterije , koje fluoresciraju na King podlozi B, a glukozu metabolišu isključivo u aerobnim uslovima; bakterija stvara levan i prouzrokuje HR duvana ali ne stvara oksidazu, pektinazu u arginin dehidrolazu (LOPAT +---+) što su odlike karakteristične za bakteriju *P. syringae*.

U pogledu patogenosti izolovanih sojeva, kao i njihovih diferencijalnih biohemijskih testova (GATT) proučavani izolati su svrstani u dve jasno izdiferencirane grupe. Prvu čine sojevi izolovani iz nekrozom zahavćenih grana trešnje, koji prouzrokuju nekrozu inokulisanih plodova trešnje, kruške i limuna, listova jorgovana i mahuna boranije. Ova grupa izolata hidrolizuje želatin i eskulin, ali ne stvaraju tirozinazu i ne metabolišu tartarate. Pomenute patogene i diferencijalne biohemijske odlike su karakteristične za *P.s.* pv. *syringae*. Nasuprot njima, sojevi izolovani iz nekrotičnih pupoljaka trešnje negativno reaguju pri pomenutim testovima patogenosti, ne hidrolizuju želatin i eskulin, ali stvaraju tirozinazu i metabolišu tartarate, što su odlike *P.s.*pv. *morsprunorum*.

REP-PCR analiza takođe ukazuje da sojevi iz nekrotičnih pupoljaka ispoljavaju identične karakteristike kao i referentni soj *P.s.* pv. *morsprunorum*. Primenom ovog metoda je takođe utvrđeno da su sojevi izolovani iz obolelih grana trešnje međusobno identični ali da se razlikuju od referentnih sojeva korišćenih u ovim istraživanjima.

Na osnovu dobijenih rezultata, tokom ovih istraživanja zaključeno je da oba patogena varijeteta *P. syringae* patogena voćaka (pv. *syrnigae* i pv. *morsprunorum*) parazitiraju trešnju u našim agroekološkim uslovima.

Ključne reči: Trešnja; *Pseudomonas syringae;* patogenost; GATT testovi; REP PCR