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ORIGINAL ARTICLE

Identification and characterization of *Pseudomonas syringae* pv. *syringae* strains from various plants and geographical regions

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Abstract

Pseudomonas syringae pv. *syringae* (Pss) constitutes a diverse group of bacterial strains that cause canker of stone fruits, blight of cereals and red streak of sugarcane. The purpose of this study was to determine how diverse Iranian strains of Pss are when they come from different hosts. We compared a total of 32 Pss strains isolated from stone fruits, barley, wheat and sugarcane from different geographical regions of Iran based on their phenotypic and molecular properties. Strains showed some variation regarding carbon and nitrogen utilization. Pss strains were similar in their protein banding patterns. Additional bands were found in sugarcane strains. Most strains showed one indigenous plasmid DNA and a few had two and some none. The genes of *syrB* and *syrD* encoding syringomycin synthesis and secretion, respectively, were amplified using specific primers in polymerase chain reaction. Syringomycin, producing strains amplified two DNA fragments of 752 and 446 bp representing *syrB* and *syrD* genes, respectively. Primer specificity was shown for Pss using various genera. Based on the results of this study, it is suggested that Pss strains from different hosts and geographical regions show diversity in phenotypic and molecular characters. It is thought that phenotypic variation is due to adaptation to specific hosts and niches for survival and pathogenicity.

Keywords: phenotype, polymerase chain reaction (PCR), protein profile, plasmid, syringomycin

Introduction

Pseudomonas syringae constitutes a diverse group of plant-associated bacteria that live either as epiphytes or plant pathogens. Some of the most important bacteria plant pathogens are listed in this genus. *Pseudomonas syringae* is a very heterogeneous group with more than 60 pathovars and nine genomospecies, belonging to RNA homology group I of *Pseudomonas*, which is part of subclass γ from class proteobacteria (Young 2010). *Pseudomonas syringae* pv. *syringae* van Hall 1902 (Pss) is considered to be one of the most important pathovars of this species with a wide host range infecting more than 180 plant species belonging to various genera (Bultreys and Kaluzna 2010). Strains of Pss cause important diseases such as bacterial canker of stone fruits, citrus blast, leaf blight of wheat and barley, sheath rot of rice, red streak of sugarcane and brown spot of bean (Kaluzna *et al.* 2010; Dariush *et al.* 2012).

Bacterial canker and gummosis of stone fruits are among the most devastating diseases of stone fruits caused by Pss and *P. syringae* pv. *morsprunorum* belonging to genomospecious 1 and 2, respectively. These pathogenic bacteria cause significant damage in nurseries and orchards. They can reduce yield between 10–20% in young orchards and even death under favorable climatic conditions (Sulikowska and Sobiczewski 2008; Bultreys and Kaluzna 2010). Disease symptoms vary in different hosts. Severity of symptoms depends on bacterial strain, host cultivar, age of the infected tree, plant tissue invaded and environmental

conditions (Gasic *et al.* 2012). The pathogen attacks different parts of stone fruit trees such as the main trunk, twigs, leaves, buds, flowers and fruits. In early spring, lesions appear on twigs underneath the infected spurs. Shoot blight and death of infected branches with gums are the result of severe infection (Bultreys and Kaluzna 2010). The bacterial disease reduces the fruit quality and quantity, and therefore the marketing value (Valencia-Botin and Cisneros-Lopez 2012). Blight of wheat and barley is caused by Pss. The late milk to soft dough stages of kernel development is the critical time for infection. In wheat and barley leaf blight, the bacterium induces small lesions and water-soaked spots on leaves. The infected leaves become necrotic and change from tan to white. Under favorable climatic conditions, the disease rapidly spreads to flag leaves during heading; blighted spots coalesce, become irregular and turn from yellow to brown. Rainfall and high humidity are necessary for infection and disease development (Valencia-Botin and Cisneros-Lopez 2012). Pss can also cause red streak of sugarcane, where yellowish-red lesions appear on any point along the leaf. Streaks are short and narrow but sometimes they can be up to 20 cm long. They turn reddish-brown and a faint yellow halo is found along the lesions. Due to a reduction in the photosynthetic rate, the yield tends to decline (Rahimian 1995).

Bacterial canker of stone fruits, red streak of sugarcane and leaf blight of wheat and barley have previously been reported in Iran (Rahimian 1995; Mohammadi *et al.* 2001; Khezri *et al.* 2010). More epidemiological information about classification and detection procedures of this bacterial pathogen is needed in order to conduct disease management. The main purpose of this study was to characterize and compare Pss strains isolated from stone fruits, cereals and sugarcane in different geographical regions in Iran based on phenotypic and molecular properties.

Materials and Methods

Bacterial strains and growth conditions

A total of 32 Pss strains from stone fruits (almond, apricot, peach and sweet cherry), sugarcane, wheat and barley, and two reference strains were characterized in this study (Table 1). Pathogenicity test on these strains had previously been carried out (Mohammadi *et al.* 2001; Khezri *et al.* 2010). Two reference strains BPIC219 and BPIC243 (Psallidas, Greece) were included in all the experiments. Each strain was streaked on nutrient agar (NA) medium, single colonies were picked up and re-streaked on the same medium to ensure purity (Lelliot and Stead 1987).

Table 1. List of Pss strains

Strain	Host plant	Province
Pss1	apricot	Charmahal Bakhtiari
Pss2-3	apricot	Fars
Pss4	apricot	Alborz
Pss5	peach	Fars
Pss6	peach	Alborz
Pss7	peach	Qazvin
Pss8	peach	Mazandaran
Pss9-10	almond	Fars
Pss11-12	almond	Charmahal Bakhtiari
Pss13	sweet cherry	Tehran
Pss14-16	sweet cherry	Alborz
Pss17-25	sugarcane	Mazandaran
Pss26-27	wheat	Charmahal Bakhtiari
Pss28-29	wheat	Fars
Pss30-31	barley	Fars
Pss32	barley	Alborz
BPIC219*	apricot	reference strain
BPIC243*	apricot	reference strain

*reference strains were provided by P.G. Psallidas, Benaki Phytopathological Institute Collection, Benaki, Greece

Biochemical and physiological characters

Pss strains were subjected to the following physiological and biochemical tests: Gram staining, KOH 3% sensitivity according to Suslow (1982), an oxidative/fermentative metabolism of glucose test according Hugh and Leifson (1953), colony morphology on sucrose peptone agar (SPA), yeast extract dextrose calcium carbonate (YDC) and sucrose nutrient agar (SNA) media, catalase, motility, LOPAT tests (levan production, oxidase, potato soft rot, arginine dehydrolase, tobacco hypersensitive reaction) (Schaad *et al.* 2001) and GATTa's group tests (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and utilization of tartrate acid) (Lelliott and Stead 1987), fluorescent pigment on KB (King'B) medium, starch hydrolysis, litmus milk, casein hydrolysis, salt tolerance (5 and 7%), gas formation from glucose, reducing compounds from sucrose, 80 hydrolysis, lecithinase, urease, nitrate reduction, ice nucleation activity and optimal growth at 4 and 41°C, 3-*keto* lactose and methyl red and acetoin (MR-VP), H_2S production from peptone and indole production according to Fahy and Hayward (1983). In addition, utilization of some sugars, amino acids, organic acids, etc., as sole carbon and nitrogen source was done by adding filtered compounds to Ayer's medium (0.5% w/v). Results were recorded up to 30 days (Schaad *et al.* 2001). Syringomycin production was tested using *Geotrichum candidum* as an indicator following Bultreys and Gheysen (1999).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Pss strains were grown on NAG (nutrient agar with 1% glucose) for 24 h at 26°C. Bacterial cells suspended in sterile distilled water (SDW) with a final optical density of 2.0 at 600 nm were centrifuged at 2,576 g for 5 min; the pellet was re-suspended in 1 ml SDW. Sample buffer (62 mM Tris-HCl, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.006% bromophenol blue, pH 6.8) was added to each tube. Samples were heated in a boiling water bath for 5 min followed by centrifugation at 6,800 g for 10 min. Samples were loaded in a SDS-polyacrylamide gel slab. The resolving gel was 12% and the stacking gel was 4%. Forty-five µl soluble protein samples were loaded in each well. Electrophoresis was carried out at a constant voltage of 100 V. Staining of the gel was done in Coomassie brilliant blue G250 (Laemmli 1970).

Plasmid extraction

Plasmid DNA extraction, purification and electrophoresis in agarose gel were done according to Maniatis *et al.* (1998). Briefly, Pss strains were individually cultured in 2.0 ml Luria-Bertani (LB) broth (pH 7.0) containing 1% (w/v) glucose in a shaker for 12 h. Plasmid DNA was extracted using the alkaline lysis method. Purified DNA samples were re-suspended in Tris-EDTA (TE) buffer (pH 7.0) and kept at –20°C. DNA electrophoresis was carried out in 1% (w/v) agarose in Tris-Borate-EDTA (TBE) buffer (pH 8.0) at a constant voltage of 80 V for 3 h. After staining with ethidium bromide, the gel was photographed using UVP gel documentation system and strains were evaluated for the presence or absence of plasmids as well as the number of plasmid.

Polymerase chain reaction (PCR)

PCR was performed according to Sorensen (1998) using Palm cycler model GP001 (Corbett Research Co., Australia). Two methods were used in preparing bacterial DNA template. In the first method, small colonies of bacteria grown on NA medium at 28°C for 2 days were touched and directly added to the reaction mixture in microtubes. In the second method, the bacterial pellet was re-suspended in 500 µl of deionized water, boiled for 15 min and then subjected to freeze-thawing at –20°C for 15 min, four times (Bultreys and Gheysen 1999). Turbidity of BPIC243, as a reference strain, was adjusted to OD_{600} nm of 1.0 and serial dilutions were prepared in SDW. Two µl from each dilution was used in PCR to determine the limit of detection. Primer pairs, each 21-nucleotide base long, used in the PCR assay included *syr*B and *syr*D. The former is designed to amplify syringomycin biosynthetic gene whereas the latter encodes for the gene involved in secretion (Sorenson *et al.* 1998). The *syr*B primer pair was as follows: B1 (5′-CTT TCC GTG GTC TTG ATG AGG-3′) and B2 (5′-TCG ATT TTG CCG TGA TGA GTC-3′). B1 and B2 primers were limited to 787- and 1539-base pair region, respectively. Thus, the PCR product for *syr*B ORF (open reading frame) was 752 bp (Sorenson *et al.* 1998). The *syr*D primer pair was as follows: D1 (5′-AAA CCA AGC AAG AGA AGA AGG-3′) and D2 (5′-GGC AAT ACC GAA CAG GAA CAC-3′). D1 and D2 primers were limited to 466- and 912-base pair region, respectively, so the PCR product for *syr*D ORF was 447 bp. The PCR mixture in a final volume of 50 µl contained 5 µl 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 pmol of each primer, 2.5 units of Taq DNA polymerase and 2 µl DNA template in sterile microtubes. The amplification program was as follows: the initial denaturation temperature for the first cycle was at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 1.5 min, primer annealing to the target DNA at 62°C for 1.5 min, and DNA extension at 72°C for 3 min. Additional extension was carried out at 72°C for 10 min (Sorenson *et al.* 1998). The final product underwent electrophoresis in 1% agarose gel using Gene Ruler™ 1 kb DNA ladder as a marker. TBE buffer (pH 8.0) at a constant voltage of 80 V for 3 h was used for electrophoresis. After staining with ethidium bromide, the gel was photographed using UVP gel documentation system. Water was used as a negative control. *Agrobacterium vitis, Bacillus subtilis, Erwinia amylovora, Pectobacterium carotovorum, Pseudomonas fluorescens, Pseudomonas viridiflava* and *Xanthomonas axonopodis* pv. *begoniae* together with standard Pss strains, BPIC219 and BPIC243, were used to determine primer specificity for *syrB* and *syrD*.

Numerical analysis

Analyses of physiological and biochemical properties and protein profile were done using the simple matching similarity coefficient and unweighted pair group with arithmetic averages using NTSYS-pc version 2.02e (Rohlf 2000).

Results

Pss colonies were white and non-slimy on SPA, cream-colored and non-slimy on YDC and round, light cream-colored and slimy on SNA media. All the strains were gram negative and obligate aerobic. In LOPAT tests, levan production and hypersensitive reaction on tobacco leave were positive but oxidase, potato soft rot and arginine dihydrolase were negative. The strains

were negative for starch hydrolysis but positive for fluorescent pigment production on KB medium after 24–48 h of incubation. Hydrolysis of casein and tween 80, motility and catalase were all positive (Table 2). In GATTa's group tests, hydrolysis of gelatin was positive, tyrosinase activity was negative and hydrolysis of aesculin and utilization of tartrate acid varied (Table 3). The Pss strains could not grow at 4°C and 41°C and 7% NaCl and showed some variation in ice nucleation activity and syringomycin production. All studied strains except Pss4, 5, 14, 30 and 31 produced syringomycin, *in vitro* (Table 3).

Pss strains were able to utilize D(+)-glucose, D(-)-fructose, D(+)-galactose, D(-)-mannose, sucrose, L-arabinose, D-sorbitol, D-xylose, *myo*-inositol, *meso*-inositol, L(-)-arabitol, D-mannitol, L-alanine, L-asparagine, L-histidine, L-proline, glycerol, citrate, malonate, succinic acid and L-arginine. Strains were varied in utilizing adonitol, raffinose, trehalose, fumarate, tartrate and L(-)-cysteine (Table 3), but none of the strains were able to utilize maltose, lactose, dulcitol, L-rhamnose, D(+)-melezitose, D(+)-cellobiose, D-arabinose, L(-)-sorbose, salicin, dextrin, glycogen, L-glycine, inulin, L-methionine, starch, oxalate, benzoate, acetate, nicotine amide, L(+)-ornithine, L-phenylalanine, DL-valine, tryptophan, ethanol or propanol.

A significant homology in banding patterns of total soluble protein was observed among Pss strains of stone fruits and cereals. SDS-PAGE analysis revealed some quantitative and qualitative differences in banding patterns between sugarcane strains with other host strains. In addition, two standard strains used in this study showed noticeable homology with banding patterns of stone fruits and cereals strains. On the other hand, sugarcane strains exhibited low molecular weights and distinct banding patterns compared to strains from other groups (data not shown).

Pss representative strains showed a considerable diversity in plasmid profiles. Some strains exhibited one plasmid band and some two plasmids, and a few showed none. These results were reproducible throughout the study. Both BPIC219 and BPIC243 had only one plasmid. Pss strains fell into three categories with respect to the number of native plasmids as shown in Table 4.

For PCR analysis, two methods were used for preparing bacterial DNA template. Both methods showed high efficiency and were used in the experiments. All syringomycin producing strains amplified two distinct DNA fragments of 725 bp and 446 bp corresponding to *syrB* and *syrD* genes, respectively (Figs 1 and 2). Pss strains 14 and 31 generated the 446 bp fragment, though neither one was a syringomycin producer (Tables 3 and 5). Both reference strains generated 725 bp and 446 bp signals.

Table 2. Phenotypic characters of Pss strains

Characteristic	Reaction
Gram staining	–
Aerobic growth	+
Levan formation	V
Oxidase	–
Potato soft rot	–
Arginine dihydrolase	–
Hypersensitive reaction	+
Fluorescent pigment	+
Gelatin hydrolysis	+
Aesculin hydrolysis	V
Starch hydrolysis	–
Tween 80 hydrolysis	+
Casein hydrolysis	+
Ice nucleation	V
Tyrosinase activity	–
Tartaric acid	V
Growth at 4°C and 41°C	–
5% NaCl tolerance	+
7% NaCl tolerance	–
Catalase	+
Nitrate reduction	–
MR-VP*	–
Phosphatase	+
Urease	+
Lecithinase	–
H_2S from peptone	–
Gas from glucose	–
Reducing compound from sucrose	+
Indole formation	–
3-*Keto* lactose formation	–
Syringomycin production	V

"+" = positive; "–" = negative; V = varied
*Methyl Red and Voges Proskauer

PCR sensitivity was tested using the syringomycin specific primer pairs. Detection limits for the reference strain BPIC243 prepared at different dilutions in deionized water were 20 and 200 cells per reaction mixture using *syr*B and *syr*D primer pairs, respectively.

In numerical analysis, four main groups were identified based on biochemical, physiological and total protein pattern (Fig. 3). About 70% similarity was observed among the three groups and two Pss standard strains. Group I included strains belonging to stone fruits and sugarcane. Most of the sugarcane strains were clustered in single clades. Wheat and barley strains with two standard strains were placed in group II. In addition, strains 2 and 8 isolated from apricot and peach were categorized in this group. Pss4, 14 and

Table 3. List of Pss strains with atypical phenotypic properties

Property	Reaction	Strain number
Syringomycin production	–	4, 5, 14, 30, 31
Tyrosinase activity	+	1, 2, 11, 14, 20, 22, 25, 27, 29
Ice nucleation activity	–	26, 28, 30
Aesculin hydrolysis	–	2, 8, 14, 26, 28
Acid production from:	+	
Adonitol	+	13, 23, 28
Cysteine	+	7, 12, 22, 29
Fumarate	+	23, 29
Raffinose	+	9, 16, 19, 20, 22, 25, 26, 27, 31
Tartrate	+	2, 29
Trehalose	+	8, 25, 27, 28

"+" = positive; "–" = negative

Table 4. Plasmid DNA profile in Pss strains

Number of plasmid DNA	Strain number
0	2, 10, 15, 18, 23, 27
1	1, 3, 5, 6, 8, 11, 12, 13, 14,17, 19, 20, 21, 26, 28, 29, 30, 31, 32
2	4, 7, 9, 16, 22, 24, 25

Table 5. List of Pss strains that did not amplify *syrB* and *syrD* genes

Genes	Strain number
syrB	4, 5, 14, 30, 31
syrD	4, 5, 30

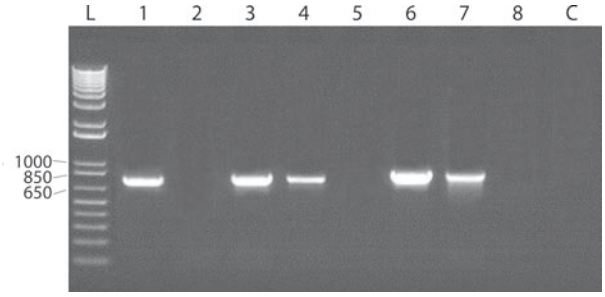

Fig. 1. Polymerase chain reaction (PCR) analysis of *syrB* gene in representative Pss strains from various host plants. L – 1.0 kb DNA ladder, (1) BPIC243 – standard strain, (2) Pss30 – barley, (3) Pss21 – sugarcane, (4) Pss12 – almond, (5) Pss31 – barley, (6) Pss13 – sweet cherry, (7) Pss27 – wheat, (8) Pss4 – apricot and C – water as a negative control

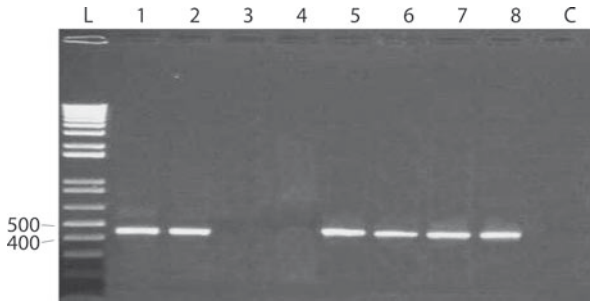

Fig. 2. Polymerase chain reaction (PCR) analysis of *syrD* gene in representative Pss strains from different host plants. L – 1.0 kb DNA ladder, (1) BPIC219 – a standard strain, (2) Pss13 – sweet cherry, (3) Pss4 – apricot, (4) Pss30 – barley, (5) Pss14 – sweet cherry, (6) Pss21 – sugarcane, (7) Pss27 – wheat, (8) Pss31 – barley and C – water as a negative control

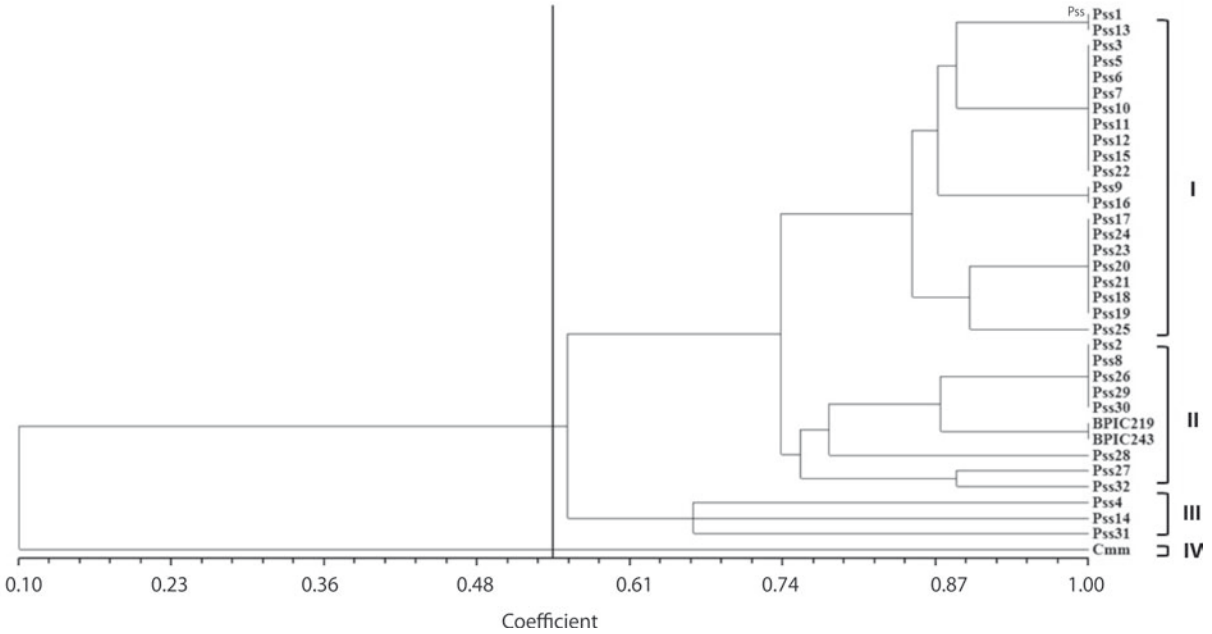

Fig. 3. Cluster analysis of *Pseudomonas syringae* pv. *syringae* strains based on the results of biochemical and physiological tests as well as protein profile (+ and –) and PCR amplification of *syrB* and *syrD* genes, using unweighted pair group with arithmetic averages. Pss1-16 from stone fruits, Pss17-25 from sugarcane and Pss26-32 from cereals. Group I – Pss strains from stone fruits and sugarcane; Group II – Pss strains from stone fruits, cereals and standard strains; Group III – Pss strains from stone fruits and cereals; and *Clavibacter michiganensis* subsp. *michiganensis* (Group IV) was used as an out-group

31 isolated from apricot, sweet cherry and barley, respectively, were placed in group III. An isolate of *Clavibacter michiganensis* subsp. *michiganensis* was used as an out-group (group IV) (Fig. 3).

Discussion

Pseudomonas syringae is a phytopathogenic proteobacterium whose host-specific pathovars collectively attack a wide range of plants including annual and perennial crops. Pss is one of the 60 pathovars that infects and causes economic losses in many plant species belonging to various families (Young 2010). In the present study, a total of 32 pathogenic strains of Pss representing different plant hosts and geographical regions were compared based on phenotypic and molecular characters.

Most phenotypic tests such as Gram staining, fluorescent pigmentation on KB medium and LOPAT were similar to strains previously reported (Mohammadi *et al.* 2001; Natalini *et al.* 2006; Ilicic *et al.* 2016). In this study, strains isolated from barley could not produce levan polymers. Gacis *et al.* (2012) reported that *P. syringae* pathovars including pv. *syringae*, pv. *morsprunorum* and pv. *persicae* isolated from stone fruits, all produced mucoid levan-type colonies on NSA medium, but they seem to produce colonies different in size and morphology. Pss strains in our study, all induced a hypersensitive reaction in tobacco which correlated with the pathogenicity test previously reported (Mohammadi *et al.* 2001; Khezri *et al.* 2010). In GATTa's group tests, Pss strains showed variation in aesculin hydrolysis and tartaric acid utilization. According to Bultreys and Kaluzna (2010), strains of *P. syringae* pv. *morsprunorum* race 1 cannot hydrolyze aesculin and gelatin but they can utilize tartaric acid and produce tyrosinase, while the results of the GATTa's group tests for other *P. syringae* pathovars are unknown (Gasic *et al.* 2012).

The pathovars of *P. syringae* are different in syringomycin production as detected using a fungal indicator such as *G. candidum*, *Saccharomyces cerevisiae* or *Rhodotorula pilimanae* or by amplifying *syrB* and *syrD* which are involved in syringomycin production and secretion, respectively (Gasic *et al.* 2012). A number of Pss strains in our study did not produce syringomycin which has been shown to correlate with pathogenicity (Mohammadi *et al.* 2001). Although some Pss strains produced syringomycin *in vitro*, they were nonpathogenic on pear, sour cherry and wheat (Sorensen *et al.* 1998). Our results showed some variation among Pss strains regarding utilization of sugars, amino acids, organic acids, etc., as sole carbon and nitrogen sources. Differences in biochemical and physiological properties as well as utilization of carbon sources in Pss strains from various hosts and geographical regions maybe related to their adaptation to specific hosts and ecological niches. In a similar study, Pss strains of stone fruits isolated from southern Iran produced acid from galactose, mannose, mannitol, sorbitol and arabinose but could not utilize lactose, maltose and tartrate (Khodaygan *et al.* 2012) which is consistent with our findings. Gasic *et al.* (2012) observed differences in acid production from inositol among *P. syringae* pv. *syringae*, *P. syringae* pv. *morsprunorum* and *P. syringae* pv. *persicae* strains.

Protein profile analysis showed a high homology in banding pattern among Pss strains with the exception of sugarcane strains that showed additional protein bands in the range of 30–45 kDa. Ravindran *et al.* (2015) reported that the number of genes identified as encoding hypothetical proteins is limited to about 16% of all Pss genes. Variations in protein profiles have been reported among Pss strains and other pathovars such as *pisi*, *tabaci*, *savastanoi, mellea* and *phaseolicola* (Soma and Samson 1991; Gonzalez-Villanuevaa *et al.* 2014) but differences among Pss isolates from different hosts are rarely seen. Rahimian (1995) reported minor differences in protein profiles between sugarcane strains and other Pss strains isolated from different geographical regions of Iran, and from Japan. Our results place sugarcane isolates in a different genotypic group from stone fruits and cereal strains. Numerical analysis based on phenotypical characters and protein profiles identified four main groups. Strains isolated from sugarcane were placed in group I but in a separate branch from wheat and barley strains (Fig. 3).

Bacterial plasmids carry genes involved in virulence, fitness, phytotoxin and hormone production, tolerance to UV radiation, resistance to antibiotics, as well as resistance to heavy metals such as copper and arsenic (Gutierrez-Barranquero *et al.* 2017). In addition, some plasmids play an important role in host-microbe interactions (Zhao *et al.* 2005). In this study, the plasmid profile among Pss strains varied from 0 to 2 bands (Table 4). The BPIC219 reference strain showed a single plasmid. Previous studies showed the existence of eight plasmid bands for *P. syringae* strains in length from 61.6 to 73.8 kbp that virulence genes, including type III secretion system effectors, were encoded on two plasmids. One of these from *P. syringae* pv. *tabaci*, encoded a wide variety of putative virulence determinants (Gutierrez-Barranquero *et al.* 2017). The complete nucleotide sequence of the five-plasmid complements of the radish and *Arabidopsis* pathogen *P. syringae* pv. *maculicola* ES4326 was determined by Stavrinides and Guttman (2004). The presence of one to several phenotypically cryptic indigenous plasmids was reported in *P. syringae* pathovars (Zhao *et al.* 2005).

Pss strains produce some nonribosomal lipodepsipeptide phytotoxins that elicit necrotic symptoms in plant tissue. Syringomycin is an important bacterial phytotoxin with a number of *syr* genes involved in regulation (*syrA*, *syrF*, *syrG* and *salA*), biosynthesis (*syrB1*, *syrB2*, *syrC* and *syrE*) and secretion (*syrD* and *syrP*) (Bender *et al.* 1999; Vaughn and Gross 2016; Adbellatif *et al.* 2017). Pss strains were compared with each other on the basis of *syrB*, *syrC* and *syrD* genes (Natalini *et al.* 2006; Gasic *et al.* 2012; Ivanovic *et al.* 2012; Ilicic *et al.* 2016). In this study, we found a correlation between syringomycin production *in vitro* and amplification of *syrB* (752 bp) and *syrD* (446 bp) genes. Pss strains that were non-syringomycin producers could not amplify both genes, except strains Pss14 and Pss31 (Tables 3 and 5). Both of which amplified a single band of 446 bp corresponding to *syrD,* but did not produce syringomycin, *in vitro.* Our results seem to agree with those of Sorensen *et al.* (1998). Bultreys and Gheysen (1999) were able to use a primer pair to detect *syrD* in three pathovars of *aptata*, *atrofaciens* and *syringae* and evaluate syringomycin and syringopeptin production by the respective strains. They also detected *P. syringae* pv. *morsprunorum* from stone fruits showing canker symptoms based on the *cft* gene. Gasic *et al.* (2012) reported that specific primers of *syrB* and *syrD* genes are suitable to detect and differentiate Pss, *P. syringae* pv. *morsprunorum* and *P. syringae* pv. *persicae*. Ilicic *et al.* (2016) reported amplification of *syrB* and *syrD* genes in all Pss strains used in their study but Bultreys and Kaluzna (2010) reported that some Pss strains do not possess both *syrB* and *syrD*. PCR using specific primers seems to be much more sensitive in detecting Pss cells than serological methods such as dot immunubinding assay (DIBA) (Khezri *et al.* 2010). We were able to detect up to 20 cells using *syrB* primers and up to 200 cells with s*yrD* primers in the PCR reaction mixture. In a previous study using polyclonal antisera against barley and sugarcane strains, Khezri *et al.* (2010) could detect 1,000 bacterial cells in 10 µl loading spot include antisera. In the current study, the specificity of using primers was showen using genomic DNA of other bacterial genera.

The results of this study suggest that a significant variation in phenotypic and molecular properties exists between Pss strains isolated from stone fruits, sugarcane and gramineous plants. These variations may refer to Pss strain adaptation to different hosts and niches during their long-lasting life. The studied strains were isolated from various geographical regions with different climate conditions. This ecological factor can also contribute to creating the diversity. In this study, both phenotypic and molecular procedures were useful in strain characterization. Additional protein bands in sugarcane strains placed them in a cluster separate from other hosts in numerical analysis. Further in-depth molecular and phylogenetic characterization of Iranian Pss strains is necessary to shed more light on how diverse these strains are, how they have evolved and adapted to various hosts, what their evolutionary relationships are, and what their pathogenicity mechanisms are.

References

Abdellatif E., Kaluzna M., Janse J.D., Sobiczewski P., Helali F., Lamichhane J.R. 2017. Phenotypic and genetic characterization of *Pseudomonas syringae* strains associated with the recent citrus bacterial blast and bacterial black pit epidemics in Tunisia. Plant Pathology 66 (7): 1081–1093. DOI: https://doi.org/10.1111/ppa.12654

Bender C.L., Alarcon-Chaidez F., Gross D.C. 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiology and Molecular Biology Reviews 63 (2): 266–292.

Bultreys A., Gheysen I. 1999. Biological and molecular detection of toxic lipodepsipeptide producing *Pseudomonas syringae* strains and PCR identification in plants. Applied and Environmental Microbiology 65: 1904–1906.

Bultreys A., Kaluzna M. 2010. Bacterial cankers caused by *Pseudomonas syringae* on stone fruit species with special emphasis on the pathovars *syringae* and *morsprunorum* race 1 and race 2. Journal of Plant Pathology 92: 21–33. DOI: 10.4454/jpp.v92i1sup.2503

Dariush S., Ebadi A.A., Khoshkdaman M., Rabiei B., Elahinia A. 2012. Characterising the genetic diversity of *Pseudomonas syringae* pv. *syringae* isolated from rice and wheat in Iran. Plant Protection Science 48 (4): 162–169. DOI: 10.17221/64/2011-PPS}

Gasic K., Prokic A., Ivanovic M., Kuzmanovic N., Obradovic A. 2012. Differentiation of *Pseudomonas syringae* pathovars originating from stone fruits. Journal of Pesticides and Phytomedicine 27 (3): 219–229. DOI: 10.2298/PIF1203219G

Gonzalez-Villanuevaa L., Arvizu-Gomeza J.L., Hernandez-Moralesb A., Aguilera-Aguirrea S., Alvarez-Moralesa A. 2014. The PhtL protein of *Pseudomonas syringae* pv. *phaseolicola* NPS3121 affects the expression of both phaseolotoxin cluster (Pht) and Non-Pht encoded genes. Microbiological Research 169 (2–3): 221–231. DOI: 10.1016/j.micres.2013.05.002

Gutierrez-Barranquero J.A., Cazorla F.M., de Vicente A., Sundin G.W. 2017. Complete sequence and comparative genomic analysis of eight native *Pseudomonas syringae* plasmids belonging to the pPT23A family. BMC Genomics 18 (1): 365. DOI: http://doi.org/10.1186/s12864-017-3763-x.

Hugh R., Leifson E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. Journal of Bacteriology 66: 24–26.

Ilicic R., Balaz J., Stojsin V., Bagi F., Pivic R., Stanojkovic-Sebic A., Josic D. 2016. Molecular characterization of *Pseudomonas syringae* pvs. from different host plants by repetitive sequence-based PCR and multiplex-PCR. Zemdirbyste-Agriculture 103 (2): 199–206. DOI 10.13080/z-a.2016.103.026

Ivanovic Z., Stankovic S., Zivkovic S., Gavrilovic V., Kojic M., Fira D. 2012. Molecular characterization of *Pseudomonas syringae* isolates from fruit trees and raspberry in Serbia. European Journal of Plant Pathology 134 (1): 191–203. DOI 10.1007/s10658-012-9978-4

Kaluzna M., Pulawska J., Sobiczewski P. 2010. The use of PCR melting profile for typing of *Pseudomonas syringae* isolates from stone fruit trees. European Journal of Plant Pathology 126 (4): 437–443. DOI: 10.1007/s10658-009-9553-9

Khezri S., Rahimian H., Ahangaran A., Mohammadi M. 2010. Comparisons of Iranian strains of *Pseudomonas syringae* pv.

syringae from various hosts with different methods. International Journal of Agriculture and Biology 12: 106–110.

Khodaygan P., Sedaghati E., Hoseinipoor A., Baghaei Ravari S. 2012. Study on canker disease of stone fruits and antibacterial effects of some plant essentials on its causal agent in Kerman province. Iranian Journal of Plant Pathology 48 (3): 303–317.

Laemmli V.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lelliot R.A., Stead D.E. 1987. Methods for the diagnosis of bacterial diseases of plants. In: "Methods in Plant Pathology". Vol. 2. (T.F. Preece, ed.). Oxford, Blackwell Scientific Publ. Ltd, UK, 216 pp.

Maniatis T., Fritisch E.F., Sambrook J. 1998. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1546 pp.

Mohammadi M., Ghasemi A., Rahimian H. 2001. Phenotypic characterization of Iranian strains of *Pseudomonas syringae* pv. *syringae* van Hall, the causal agent of bacterial canker disease of stone fruits trees. Journal of Agricultural Science and Technology 3: 51–65.

Natalini E., Rossi M.P., Barionovi D., Scortichini M. 2006. Genetic and pathogenic diversity of *Pseudomonas syringae* pv. *syringae* isolates associated with bud necrosis and leaf spot of pear in a single orchard. Journal of Plant Pathology 88 (2): 219–223. DOI: http://dx.doi.org/10.4454/jpp.v88i2.866

Rahimian H. 1995. The occurrence of bacterial red streak of sugarcane caused by *Pseudomonas syringae* pv. *syringae* in Iran. Journal of Phytopathology 143 (6): 321–324. DOI: 10.1111/j.1439-0434.1995.tb00268.x

Rohlf F.J. 2000. NTSYSpc: Numerical taxonomy and multivariate analysis system. Version 2.1. Exeter Software Setauket, NY, USA.

Schaad N.W., Jones J.B., Chun W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd ed. St. Paul, MN, APS Press, 373 pp. DOI: https://doi.org/10.1046/j.1365-3059.2001.00635.x

Soma A., Samson R. 1991. Protein and enzymatic criteria for the characterization of phytopathogenic *Pseudomonas fluorescens*. Comptes Rendus De l'Academie Des Sciences. Serie III, Sciences De La Vie 312 (5): 203–208.

Sorensen K.N., Kim K.H., Takemoto J.Y. 1998. PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. Applied and Environmental Microbiology 64 (1): 226–230.

Stavrinides J., Guttman D.S. 2004. Nucleotide sequence and evolution of the five-plasmid complement of the phytopathogen *Pseudomonas syringae* pv. *maculicola* ES4326. Journal of Bacteriology 186 (15): 5101–5115. DOI: 10.1128/JB.186.15.5101-5115.2004

Sulikowska M., Sobiczewski P. 2008. *Pseudomonas* spp. isolated from stone fruit trees in Poland. Zemdirbyste-Agriculture 95 (3): 166–170.

Suslow T.V., Schroth M.N., Isaka M. 1982. Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining. Phytopathology 72: 917–918.

Ravindran A., Jalan N., Yuan J.S., Wang N., Gross D.C. 2015. Comparative genomics of *Pseudomonas syringae* pv. *syringae* strains B301D and HS191 and insights into intra pathovar traits associated with plant pathogenesis. Microbiology Open 4 (4): 553–573. DOI: 10.1002/mbo3.261

Valencia-Botin A.J., Cisneros-Lopez M.E. 2012. A review of the studies and interactions of *Pseudomonas syringae* pathovars on wheat. International Journal of Agronomy 4 (1): 5. DOI: http://doi.org/10.1155/2012/692350.

Vaughn V.L., Gross D.C. 2016. Characterization of *salA*, *syrF*, and *syrG* genes and attendant regulatory networks involved in plant pathogenesis by *Pseudomonas syringae* pv. *syringae* B728a. PLoS One: e0150234. DOI: http://doi.org/10.1371/journal.pone.0150234.

Young J.M. 2010. Taxonomy of *Pseudomonas syringae*. Journal of Plant Pathology 92 (1, Supplement): S5–14. DOI: 10.4454/jpp.v92i1sup.2501

Zhao Y., Ma Z., Sundin G.W. 2005. Comparative genomic analysis of the pPT23A plasmid family of *Pseudomonas syringae*. Journal of Bacteriology 187 (6): 2113–2126. DOI: 10.1128/JB.187.6.2113-2126.2005