

Phenotypic Characterization of Iranian Strains of *Pseudomonas syringae* pv. *syringae* van Hall, the Causal Agent of Bacterial Canker Disease of Stone Fruit Trees

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ABSTRACT

A total of twenty seven bacterial strains were isolated from cankerous tissues of apricot, nectarine, peach, plum, sour cherry and sweet cherry trees in Tehran province and identified as *Pseudomonas syringae* pv. *syringae* (Pss), the causal agent of the bacterial canker disease, on the basis of LOPAT (levan production, oxidase test, potato rot, arginine dihydrolase and tobacco hypersensitive reaction) and GATTA's (gelatin liquefaction, aesculin hydrolysis, tyrosinase activity and Na-tartrate utilization) group tests. Pss strains showed slight differences in morphology, phenotypic (biochemical and physiological) characteristics, serological properties, plasmid DNA and cellular protein profiles and antibiogram. They were divided into three distinct groups based on hippurate and formate utilization which was correlated with protein profile in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The virulence of Pss was significantly associated with the degree of necrosis on immature sweet cherry fruits and the rate of *in vitro* syringomycin production.

Keywords: Bacterial canker disease, *Pseudomonas syringae* pv. *Syringae*, Stone fruit trees, Syringomycin.

INTRODUCTION

Pseudomonas syringae pv. *syringae* van Hall is the causal agent of bacterial canker disease of stone fruit trees. The disease is also known as gummosis, blossom blast, dieback, spur blight and twig blight (Hattingh and Roos, 1995). It is one of the most devastating diseases of stone fruit trees with a disease loss of 10-75% in young orchards (Agrios, 1988). This disease was reported for the first time on apricot trees in Iran (Bahar *et al.*, 1985). Subsequently, the causal agent of bacterial canker on apricot, peach and plum trees was identified as *P. syringae*

pv. *syringae* (Shamsbakhsh and Rahimian, 1997).

Disease symptoms include canker development on shoots and at the base of spurs and its progression upward accompanied with gum exudation early in the growing season (Hattingh and Roos, 1995). The pathogen attacks twigs, buds, flowers, leaves and fruits. In the early spring, dark brown sunken lesions appear on twigs beneath the infected spurs. Severe infection of twigs results in shoot blight and death of the infected branches with gums often appearing from cankered regions on the limbs (Goto, 1992).

There are two pathovars in *P. syringae*: pv. *syringae* van Hall which causes canker

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disease on all commercially grown stone fruit tree species and pv. *morsprunorum* (Wormald) Young *et al.* that primarily infects sweet cherry, sour cherry and plum. Both pathovars belong to RNA homology group I of the genus *Pseudomonas* (Palleroni, 1984). There are several methods to differentiate the two pathovars: a) the use of GATT a and b) DNA homology study (Hattingh and Roos, 1995).

In addition to sharing bacteriological properties with *P. syringae* (CMI, 1965), the pathovar *syringae* possesses the following characteristics: levan positive, gelatin, aesculin and arbutin hydrolysis positive; betaine, erythritol, D-gluconate, *meso*-inositol, mannitol, caprylate, sorbitol, trigonelline and D-xylose utilization as a sole carbon source positive. Utilization of anthranilate, DL-homoserine, L(+) tartrate is negative. Acetate, L-ascorbate, L-histidine, DL-hydroxy-buturate, DL-lactate and D(-) tartrate utilization vary (Bradbury, 1986; Young and Triggs, 1994). *P. syringae* is a very heterogenous group. Young (1991) has suggested eleven biochemical tests for the identification of Pss. He believed that (a) some isolates have a little homology with Pss and may be classified as a new species, (b) others could be similar or different due to the host-range specificity and (c) isolates that have distinct phenotypic characteristics and a broad host range such as the causal agent of lilac disease but their host range is not limited to lilac.

Availability of biochemical diagnostic kits has made the identification of Pss easier and highly reliable. BIOLOG system using 95 carbon sources has confirmed that *P. syringae* is heterogeneous and specific pathovars are differentiated based on a single metabolic pattern (Rudolph, 1995). *P. syringae* pathovars have DNA homology at 40-100%, whereas this is between 95-100% among the isolates of one particular pathovar (Palleroni *et al.*, 1972). Isozymic profiles have distinguished *syringae* and *morsprunorum* pathovars from each other (Rudolph, 1995). *P. syringae* pathovars have been characterized according to the fatty

acid profile (Rudolph, 1995), antigenic specificity (Lucas and Grogan, 1969), phage typing (Anderson, 1980; Okabe and Goto, 1963) and syringomycin production (Xu and Gross, 1988). The potential for syringomycin synthesis has been correlated with the degree of Pss virulence (Enderet and Ritchie, 1984). Syringomycin, as a phytotoxin, induces tissue necrosis in the host plant by disrupting physiological functions of plasmalemma and causing cell collapse (Xu and Gross, 1988).

This study was undertaken (a) to determine the phenotypic properties of Iranian strains of *P. syringae* pv. *syringae* isolated from various stone fruit trees and (b) to investigate whether the degree of *in planta* necrosis is associated with *in vitro* syringomycin production.

MATERIALS AND METHODS

Bacterial Isolation from Tree Cankers

P. syringae pv. *syringae* was isolated from cankerous tissues of stone fruit trees such as apricot, peach, plum, sour cherry, sweet cherry and nectarine in Karaj region of Iran according to the method of Roos and Hattingh (1986). Branches and twigs showing cankerous symptoms were surface-sterilized in 70% ethanol and then areas adjacent to the cankerous tissues were cut into small pieces of 2-5 mm in length and placed in 5 ml sterile distilled water in test tubes. Test tubes were shaken vigorously every 30 min for 1 to 2 hrs. A loopful of bacterial suspension was streaked on CMB agar medium (King's B and 50 µg/ml cyclohexamide) and nutrient agar containing 5% sucrose.

Bacterial Isolation from Leaves

Leaf tissues showing necrotic lesions and blight symptoms were washed in water and several leaf pieces were homogenized in sterile distilled water in mortar. A loopful of homogenate was streaked out on CMB and

nutrient agar media. Petri plates were incubated at 25°C for two days. Bacterial colonies that fluoresced under UV light or produced levan polymer on NAS medium (nutrient agar and 5% sucrose) were selected and grown on King's B (KB) medium (King *et al.*, 1954).

Bacterial Single Colony Isolation and Maintenance

Colonies were streaked out on nutrient agar medium and single colonies were selected. Colonies were then cultured on KB slants in tubes for 48 hrs and their surfaces being covered with sterile liquid paraffin. In addition, a suspension of each bacterial strain was kept in 1.0 ml sterile distilled water at 4 °C or in a sterile mixture of 20% glycerol and 100 mM CaCl₂ in Eppendorf tubes at -20 °C.

Pathogenicity Test

Pathogenicity test on apricot leaves: Fully developed young apricot leaves were removed from young shoots with 20-25 cm length and placed in plastic bags for 48 hrs prior to inoculation according to Roos and Hattingh (1987a). A 10 µl droplet of bacterial suspension (1×10^7 cells/ml) was placed in the central portion of a leaf and the leaf blades were gently pressed against each other to bring about mild injury. Control was treated with sterile distilled water instead. Leaves were kept in plastic bags for 48 hrs. The results were recorded from four days after inoculation.

Pathogenicity test on immature sweet cherry fruits: Immature sweet cherry fruits were picked 2-4 weeks before ripening and surface-sterilized with 95% ethanol. Ten µl of bacterial suspension culture (1×10^7 cells/ml) was injected into each cherry fruit based on the methods of Jones (1971) and Lattore and Jones (1979). Control fruits were injected with sterile distilled water. Cherry fruits were placed in a moist Petri dish at

25°C. Symptoms appeared as depressed brown spots on fruits after 48-72 hrs.

Pathogenicity test on sweet cherry shoots: Young shoots from cherry tree were cut with a sterile scalpel and inoculated with 10 µl of bacterial suspension (1×10^6 cells/ml) (Jones and Lattore, 1979). The inoculation site was covered with parafilm (to prevent bacterial inoculum from evaporating) and the plants were kept in a greenhouse for upto a month.

Phenotypic characterization of Pss strains: Pss strains were characterized based on the following tests: Gram test in 3% KOH (Susslow), oxidative/fermentative test (Hugh & Leifson), hypersensitive reaction in tobacco leaves, fluorescent pigment production on KB medium, oxidase test, levan production, maceration of potato slices, catalase, urease, gelatin liquifaction, litmus milk, salt tolerance (5% and 7%) and gas formation from glucose. In addition, tests for arginine dehydrolyase, hydrogen sulfide production from peptone, reducing substance from sucrose, tyrosinase casein hydrolase, nitrate reduction indole production, 2-keto gluconate oxidation lecithinase, MR-VP tests, starch hydrolysis phenylalanine deaminase, aesculin and tween80 hydrolysis, ice nucleation activity and optimal growth temperature on Ayer medium were also performed. Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily upto 28 days (Hildebrand *et al.*, 1988). Each test was repeated twice except for those strains that showed variable results in which the corresponding test was repeated three times.

Serological Tests

Antiserum production: *P. syringae* pv. *syringae* strain #3 was grown on KB medium for 24 hrs and a cell suspension was prepared in saline solution (0.85% NaCl). The bacterial suspension was autoclaved at 121°C for 30 min. and then centrifuged at 15,000×g for 10 min. The pellet was washed in saline solution and a suspension of 1×10^{10} cells/ml was mixed with an equal



volume of incomplete Freund adjuvant. The mixture was injected intramuscularly into rabbit two times at seven days apart and three more injections administered subcutaneously in the ear without adjuvant each 7 days apart. Ten days after the last injection, the animal was sacrificed and the antiserum was clarified (Jones *et al.* 1983).

Ouchterlony double diffusion test: Agarose gel was prepared according to Hildebrand *et al.* (1988). The gel solution containing 0.75% agarose (Merk Co.), 0.02 g sodium azide, 0.85 g NaCl and 2 g Mg Cl₂ in 100 ml distilled water was melted and poured into plastic Petri dishes of 9 cm diameter and kept at 4°C. Small wells (3 mm in dia.) were cut at the periphery and center of each plate. Twenty µl antiserum was placed in the central well and an equal amount of autoclaved bacterial antigen in the peripheral wells. The plates were incubated at 4°C for three days. Following the appearance of precipitin bands, the agarose plate was washed in phosphate-saline buffer for 2-18 hrs in order to remove non-reacted antigen and antibody. The gel was subsequently washed in double distilled water for 1-2 hrs and dehydrated with several layers of filter paper under pressure. After relative drying, the gel was stained with coomassie brilliant blue G250 in water, methanol and acetic acid (5:5:1) for 20-30 min. Destaining was done in the same solution without the dye. The precipitin bands were photographed.

Antibiotic Sensitivity Test: Pss strains were tested for antibiotic sensitivity using antibiogram disks. A bacterial suspension was evenly spread on nutrient agar medium containing 2% sucrose. Two disks for each antibiotic were placed on medium. After 48 hrs of incubation at 28°C, inhibition zone was measured.

SDS-PAGE

Electrophoresis of soluble proteins was carried out in a discontinuous SDS-polyacrylamide gel according to the method of Laemmli (1970) with some modifications

as described by Rahimian (1991). Pss strains were grown on KB medium at 24°C for two days. A bacterial suspension in distilled water was prepared in Eppendorf tube and centrifuged at 15,000 xg for 2 min. The pellet was washed and resuspended in sterile distilled water. The cell suspension optical density was adjusted to 1.0 at 630 nm. Each sample was mixed with 5x sample buffer [sample buffer: 63 mM Tris-HCl (pH 6.8), 10 % (v/v) glycerol, 2.0% (w/v) SDS and 0.25% (w/v) bromophenol blue] and heated at 95°C for 5 min. To each 950 µl sample buffer, 50µl of 2-mercaptoethanol, as a reducing agent, was added just before boiling. Samples were centrifuged at 15,000 x g for 10 min. Fifty µl of soluble proteins was loaded in each well in a 13×17 cm polyacrylamide slab with 0.75 mm thickness. Proteins were fractionated in 10% resolving gel at a constant current of 20 mAmps for four hrs. The gel was stained in methanol, water and acetic acid (5:5:1) containing 0.5% coomassie brilliant blue G250 overnight and destained in the same solution without dye. The gel was kept in 7% acetic acid.

Plasmid DNA Extraction

Plasmid DNA was extracted and purified using the alkaline lysis procedure as described by Maniatis *et al.* (1982). Pss strains were grown in 5 ml Luria broth (5 g NaCl, 2.5 g nutrient broth, 5 g yeast extract, 10 g tryptone in one liter of distilled water) in tubes on a rotary shaker at 28°C overnight. Bacterial culture was centrifuged at 15,000x g for 5 min. The pellet was resuspended in lysis buffer (50 mM glucose, 25 mM Tris buffer (pH 8.0) and 10 mM EDTA), mixed with alkaline solution (0.2 N NaOH and 1% SDS) and acidified in 3 M potassium acetate buffer (pH 5.4). After centrifugation, the supernatant was purified with phenol: chloroform: isoamylalcohol (24:24:1), and chloroform isoamylalcohol and then centrifuged. The aqueous layer was precipitated with 96% ethanol and washed in 70% ethanol.

The DNA pellet was resuspended in 25 mM Tris- EDTA buffer (pH 8.0).

Agarose Gel Electrophoresis of Plasmid DNA

Electrophoresis of plasmid DNA was performed in 0.8% (w/v) agarose gel in Tris-acetate (94 mM) buffer (pH 8.3) containing 10 µl ethidium bromide (1 mg/liter) as described by Maloy (1990). Ten µl DNA sample was mixed with 2 µl of 5x sample buffer [sample batter: 63mM Tris-base (pH 8.3), 10% (v/v) glycerol and 0.25% (w/v) bromophenol blue-xylene cyanol] and placed into agarose well. DNA electrophoresis was carried out in Tris-acetate buffer at a constant voltage of 55 volts for 3 hrs. DNA bands were photographed on UV- transilluminator (Sigma Chemical Co., St. Louis, MO, USA) using a Polaroid film.

Evaluation of Pss Virulence

The virulence of Pss strains from cherry (3), apricot (2), peach (2), plum (1), prune (1) and nectarine (1) was evaluated according to Enderet and Ritchie (1984) with some modification as follows:

Evaluation of Pss virulence on immature sweet cherry fruits: Immature sweet cherry fruits of a similar size from a single tree were surface-sterilized with 0.5% (v/v) sodium hypochlorite. A suspension (1×10^7 cells/ml) of each Pss strain was prepared from a 48 hr-old culture in a sterile phosphate buffer. Immature cherry fruits were inoculated with an inoculation needle 2 mm deep. This was done in triplicates, sterile distilled water being used as control. The diameter of each necrotic region was measured after four days, the means being compared in a complete randomized design.

Evaluation of Pss virulence based on the degree of syringomycin production: Pss strains in triplicates were spotted on PAD medium containing 1 mg/ml apricot shoot extract based on the refined method of Gross (1985)

in a complete randomized design. The culture plates were kept at 25°C for four days. The bacterial colonies were wiped off from each plate and the agar surface sterilized using acetone. After a complete evaporation of acetone, a conidial suspension of *Geotrichum candidum* was sprayed on each culture plate and the inhibition zone measured after 48 hrs.

RESULTS

Phenotypic Characteristics

Bacterial colonies grown on NSA medium for 48 hrs were 2 mm in diameter, light cream with entire margins, slimy and fluorescent. They were compared with respect to physiological, biochemical, serological, protein and plasmid DNA profiles and anti-biogram.

A total of 27 bacterial strains were isolated from sweet cherry, sour cherry, apricot, peach, plum and nectarine trees and characterized phenotypically (Tables 1 & 2). All 27 strains were Gram and oxidase negative, and unable to utilize glucose under anaerobic conditions. None of the strains were able to macerate potato slices, produce reducing compounds from sucrose or show tyrosinase, lecithinase, arginine dihydrolase activity or produce gas from glucose and sodium tartrate. All strains were catalase positive and capable of hydrolyzing gelatin, aesculin, tween 80 and casein. None of the strains were able to produce indole, reduce nitrate and oxidize 2-keto-gluconate. Strains were all able to produce syringomycin and urease and showed ice nucleation activity. Litmus milk test became alkaline and NSB medium turned yellow.

All strains were able to utilize arginine, ascorbate, L-asparagine, biotin, butyrate, citrate, D (-) fructose, fumarate, D (+) galactose, galacturonate, gluconate, D-glucose, L-glutamic acid, glycerol, histidine, lactate, L-leucine, malonate, D-mannitol, D-mannose, D (+) Melezitose, Meso-erythritol, Myo-inositol, D-raffinose, D- bitol and sucrose.

**Table 1.** Origin, host plant and location of *P. syringae* pv. *syringae* strains used in this study.

Strain #	Host plant	Location	Origin
1	Peach	Baraghan, Karaj	This study
2	"	"	"
3	Sweet cherry	Baraghan, Sâvejbalagh	"
4	"	"	"
5	"	"	"
6	"	"	"
7	"	"	"
8	"	"	"
9	"	"	"
10	Peach	Takistan	"
11	"	"	"
12	"	"	"
13	"	Sangan	"
14	"	Karaj	"
15	"	Shahriar	"
16	Sweet cherry	Tehran	"
17	"	"	"
18	"	Damavand	"
19	"	Karaj	"
20	Apricot	"	"
21	Sweet cherry	Moghan	Dr. Mitra Mazarei
22	Plum	Damavand	This study
23	Sweet cherry	Kurdestan	"
24	Sour cherry	Shahrestanak	"
25	Apricot	"	"
26	Nectarine	Karaj	"
27	Sweet cherry	Braghan, Savejbalagh	"

None of the strains were capable of utilizing adenine, adonitol, L-alanine, L (-) arabinitol, D (+) cellobiose, L -cysteine, L (-) cysteine, dextrin, ducitol, glycine, L- homoserine, inulin, isoleucine, lactose, maltose, L -methionine, nicotinamide, niacin, D- ornithine, oxalate, DL -phenylalanine, L- proline, propionate, ribose, L -rhamnose, salicin, L-sorbose, L-tartrate, taurocholic acid, thiamin chloride, DL-tryptophan, L- tyrosine, DL-valine and L-valine. Only three strains utilized hippurate and eight strains formate.

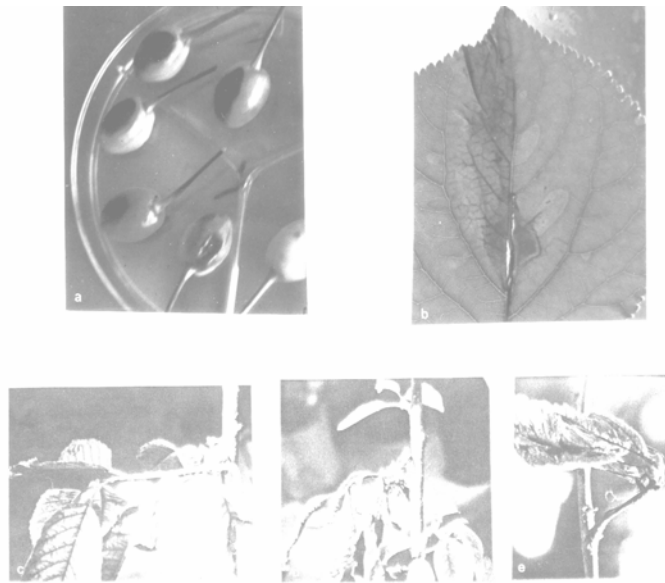
Antibiogram Test

Pss strains were tested in duplicates for sensitivity toward 30 different antibiotics (Table 3). They were classified into three groups: resistant (without an inhibition

zone), relatively sensitive (inhibition zone with a diameter less than 20 mm) and sensitive (inhibition zone greater than 20 mm in diameter).

Pathogenicity Test

All 27 Pss strains caused dark brownish spots with depressions on the surface of immature cherry fruits 2-3 days after inoculation, whereas a slight discoloration was observed in the control (Fig. 1). Pss strains also caused water-soaked spots and necrosis in the midrib region on apricot leaves after four days. These symptoms did not occur in the control. Further, inoculation of young shoots on cherry trees with individual Pss strains caused chlorotic spots that eventually became necrotic and dried.



Figur1. *Pseudomonas syringae* pv. *syringae* pathogenicity test on stone fruit trees. Pathogenicity test on (a) immature sweet cherry fruits; (b) apricot leaf four days after inoculation and on sweet cherry shoots 3 (c). 10 (d) and 30 (e) days post-inoculation.

Ouchterlony Double Diffusion Test

Ouchterlony double diffusion test showed that all 27 strains of Pss were serologically homologous forming a single precipitin band with the polyclonal antiserum prepared in rabbit against Pss strain #3 from sweet cherry (Fig. 2). The Pss antiserum was unable to identify *P. syringae* pv. *mori* from mulberry.

Plasmid DNA Profile

All 27 Pss strains showed a single plasmid DNA band in agarose gel electrophoresis (Fig.3). There was no difference in migration pattern of the plasmid DNA among the strains tested. Pss plasmid DNA seems to be similar in size as compared to that from *Ralstonia solanacearum*.

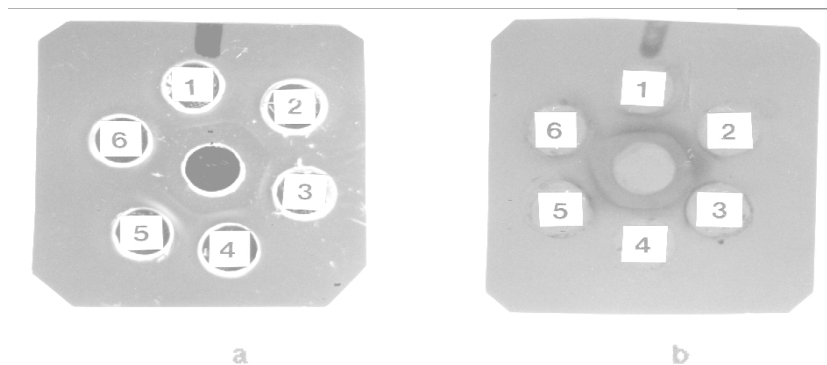


Figure 2. Ouchterlony double diffusion test in agarose gel. The antiserum prepared against Pss isolate # 3 was charged in the central well and peripheral wells were loaded with antigen from Pss strains# 3 (1), 9(2), 15(3), 17(4), 23(5) and *P. S. Pv. mori* (6) from mulberry. Precipitin bands before (a) and after (b) staining with coomassie brilliant blue G250



**Table 3.** Antibiotic sensitivity of *P. syringae* pv. *syringae* strains used in this study.

Antibiotic	µg/disk ^a	Resistant ^b	Relatively sensitive ^c	Sensitive ^d
Amoxicillin	25	+	-	-
Ampicillin	10	+	-	-
Carbenicillin	100	+	-	-
Cefazolin	30	+	-	-
Cephalexin	30	+	-	-
Cephalotin	30	+	-	-
Clindamycin	2	+	-	-
Glucose acillin	5	+	-	-
Lincomycin	2	+	-	-
Nitrofurantoin	300	+	-	-
Oxacilin	1	+	-	-
Penicillin	10	+	-	-
Sefradin	30	+	-	-
Seftysorkims	30	+	-	-
Trimethoprim sulfaxasol	-	+	-	-
Vancomycin	30	+	-	-
Colistin sulfate	10	-	+	-
Erythromycin	15	-	+	-
Gentamycin	10	-	+	-
Nalidixic acid	30	-	+	-
Neomycin	30	-	+	-
Rifampicin	30	-	+	-
Amikacin	30	-	-	+
Chloramphenicol	30	-	-	+
Furasolidon	15	-	-	+
Kanamycin	30	-	-	+
Doxycycline	30	-	-	+
Oxy-tetracycline	30	-	-	+
Streptomycin	10	-	-	+
Tobramycin	10	-	-	+

^a Except for penicillin, amoxicillin and ampicillin in which the concentration was in unit per disk.

^b No inhibition zone.

^c Inhibition zone with a diameter less than 20 mm.

^d Inhibition zone with a diameter equal or greater than 20 mm.

Protein Profile

Seventeen Pss strains isolated from various hosts showed an identical protein pattern in SDS-PAGE. Pss strains 23 and 25 isolated from sweet cherry and apricot trees, respectively, had slightly different protein profiles from those of the remaining strains. This variation was observed in low molecular weight protein bands. *P. syringae* pv. *tomato* and pv. *morsprunorum* showed slightly different protein patterns from those of Pss (Fig. 4).

Table 4. Analysis of variance on the degree of virulence of Pss isolates based on syringomycin production and the necrotic lesion formation on immature sweet cherry fruits.

Degree of freedom	Mean of squares	
	Syringomycin	Necrotic lesion size
Strain # 9	24.152 **	17.259 **
Error 20	3.267	0.692

** Significantly different at the level of 0.1%

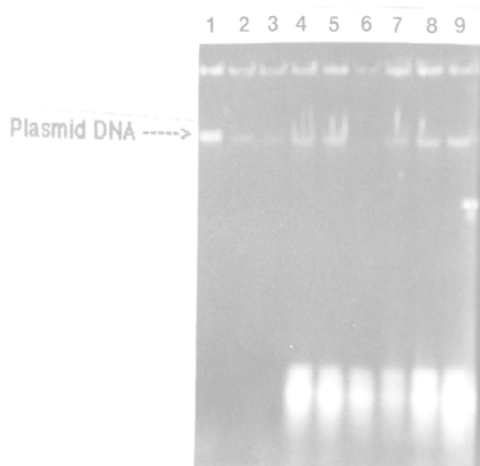


Figure 3. Agarose gel electrophoresis of *P. syringae* pv. *syringae* plasmid DNA profile. From left to right, plasmid DNA band from *R. solanacearum* (lanes 1-3) and Pss strains (lanes 4-9).

Evaluation of Pss Virulence

The virulence of Pss strains was evaluated based on the degree of syringomycin production in agar medium and the diameter of necrotic spot induced on immature cherry fruits. The means were statistically distinct and significantly different at 1% level in two

completely randomized designs (Table 4 and Fig. 5). The correlation between the means of treatments in two separate experiments was around 63% which shows that Pss virulence on immature cherry fruits is directly related to syringomycin production.

DISCUSSION

Based on GATTa scheme, we identified all 27 bacterial strains isolated from cankerous tissues of various stone fruit trees as *P. syringae* pv. *syringae* (Table 2). Pss strains are $G^+A^+T^-Ta^-$, whereas Psm strains are $G^-A^-T^+Ta^+$ (Lattore and Jones, 1979). Although, it has been reported that there may be some intermediate strains diverging from one or more of the four tests (Hattingh and Roos, 1995), Pss strains in this study were all positive for gelatin liquefaction and aesculin hydrolysis while being negative for tyrosinase activity and tartrate utilization. All Pss strains produced fluorescent pigments on King's B medium and were negative for arginine dihydrolase and oxidase tests. Pss strains were levan producers and formed whitish to cream-colored, dome-shaped colonies on nutrient sucrose medium and a yellow supernatant fluid in SNB. They

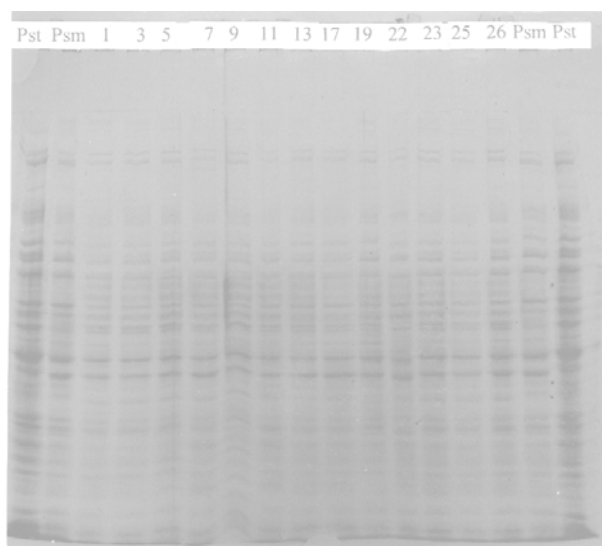


Figure 4. SDS-PAGE analysis of total soluble proteins from Pss strains. Pst, *P. syringae* pv. *tomato*; Psm, *P. syringae* pv. *Morsprunorum*.



also produced syringomycin and showed ice-nucleating activity, both of which are mostly absent in pathovar *morsprunorum*. These results are consistent with those previously reported by Endert and Ritchie (1984), and Latorre and Jones (1979).

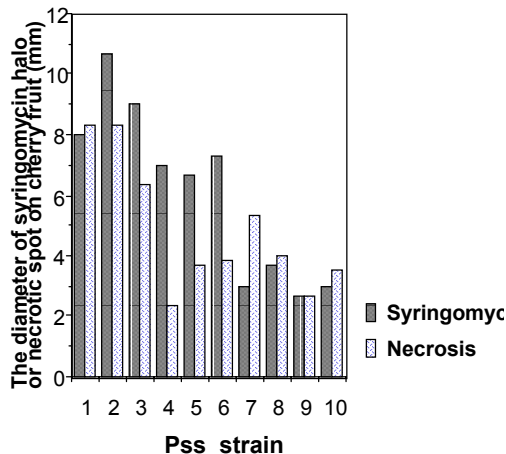


Figure 5. The degree of virulence of *P. syringae* pv. *syringae* strains based on syringomycin production and the extent of necrosis on immature sweet cherry fruits. Numbers 1-10 represent Pss strains # 1, 3, 5, 9, 12, 15, 17, 19, 25 and 27, respectively.

Pss strains were divided into three groups based on carbohydrate utilization: (a) strains #18, 19 and 20 that were able to utilize hippurate, (b) strains # 9, 10, 15, 16, 17, 23, 26 and 27 that were formate positive and (c) strains that utilized neither one, as the sole carbon source (Table 2). Pss strains in this study were different from those reported by Shamsbakhsh and Rahimian (1997) with regard to D-raffinose utilization. Further, these strains were at variance with the type species (CMI, 1965) and that reported by Banapour *et al.* (1990) from Damavand region in Iran in terms of D(+)-melezitose utilization. All 27 Pss strains in this investigation were positive regarding these two carbon sources.

All 27 strains of Pss were able to induce HR on tobacco leaves, a test which is considered to be a reliable criterion for testing pathogenicity (Roos and Hattingh, 1983). Further, we observed that all Pss strains

caused dark brown and sunken lesions on immature sweet cherry fruits 2-3 days after inoculation. However, Latorre and Jones (1979) warned that these tests alone may be insufficient to detect pathogenicity in Pss strains. Further, syringomycin production may not correlate with pathogenicity. In this study, the degree of virulence among Pss strains was related to the level of *in vitro* syringomycin production and the extent of necrosis on immature sweet cherry fruits at 63% (Figure 5). These results are in agreement with those obtained by Devay *et al.* (1968). This may indicate that cherry fruits are capable of detecting Pss pathogenicity as suggested by Endert and Ritchie (1984). They also observed induction of lesions on cherry fruits correlated with pathogenicity of highly virulent strains. Further, these authors concluded that inoculation of peach seedling cotyledons seems to be a sensitive bioassay for detecting pathogenicity of Pss strains.

The antiserum prepared from heat-killed cells of Pss strain #3, was pathovar-specific and able to distinguish Pss strains from the pathovar *mori*. The latter failed to show a precipitin band in the double diffusion test (Figure 2). Lovrekovich *et al.* (1963) revealed specific antigenic determinants in *P. tabaci*, *P. lachrymans* and *P. morsprunorum* using Ouchterlony double diffusion method. SDS-PAGE analysis could distinguish *P. syringae* pathovars and the variation among Pss strains was correlated with the phenotypic characteristics including hippurate utilization. Palmer and Cameron (1971) confirmed that electrophoretic protein analysis is a very efficient technique in distinguishing *P. syringae* pathovars from each other. All 27 Pss strains, in this study, showed a single plasmid DNA band in agarose gel electrophoresis (figure 3). Sundin *et al.* (1989) also detected the presence of a single plasmid DNA with MW of 46-73 kb in copper resistant strains of Pss.

Although, Pss strains used in this study came from various geographical locations within Tehran province, they all shared common phenotypic properties with the exception of a minor variation in carbon utili-

zation. Roos and Hattingh (1987b) reported significant differences between groups of strains within Pss in South Africa suggesting a heterogeneous population of Ps clustered around Pss. It would be more interesting to study phenotypic characteristics of a large population of Iranian strains of Pss from various host plants in different regions.

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REFERENCES

1. Agrios, G. N. 1988. Plant Pathology. 3rd ed. Academic Press. 803 pp.
2. Anderson, A. J. 1980. Unique Aspects of the Cell Surface Polysaccharide of *Pseudomonas phaseolicola* as Demonstrated by Bacteriophage Specificity. *Can. J. Microbiol.*, **26**: 1422-1427.
3. Bahar, M., Mojtahedi, H., and Akhiani, A. 1985. Bacterial Canker Disease of Apricot in Esfahan. *Iran. J. Plant Pathol.*, **18**: 58-68.
4. Banapour, A., Zakiee, Z., and Amani, G. 1990. Isolation of *Pseudomonas syringae* pv. *syringae* from Sweet Cherry Trees in Tehran Province, Iran. *J. Plant Pathol.*, **26**: 67-72.
5. Bradbury, J. F. 1986. Guide to Plant Pathogenic Bacteria, CAB. pp. 110-176.
6. CMI. 1965. *Pseudomonas syringae*. Descriptions of Pathogenic Bacteria, No. 46.
7. Devay, J. E., Lukezic, F. L., Sinden, S. L., English, H., and Coplin, D. L. 1968. A Biocide Produced by Pathogenic Isolates of *P. syringae* and Its Possible Role in the Bacterial Canker Disease of Peach Trees. *Phytopathology.*, **58**: 95-101.
8. Enderet, E., and Ritchie, D. F. 1984. Detection and Pathogenicity Measurement of Virulence and Determination of Isolate Variation in *Pseudomonas syringae* pv. *Syringae*. *Plant Dis.*, **68**: 677-680.
9. Goto, M. 1992. Fundamentals of Bacterial Plant Pathology, Academic Press, Inc. New York. 342 pp.
10. Gross, D. C. 1985. Regulation of Syringomycin Synthesis in *Pseudomonas syringae* pv. *Syringae* and Defined Condition for Its Production. *J. Appl. Bacteriol.*, **58**: 167-174.
11. Hattingh, M.J., and Roos, I. M. M. 1995. Bacterial Canker. In: "Compendium of Stone Fruit Diseases." (Eds): Ogawa, J.M., Zehr, E.I., Bird, G.W., Ritchie, D.F., and Uymoto, J.K., The Am. Phytopathol. Soc. Press, St. Paul, MN, USA, pp. 48-50.
12. Hildebrand, D.C., Schroth, M.N., and Sands, D.C. 1988. *Pseudomonas Syringae*. In: "Laboratory Guide for the Identification of Plant Pathogenic Bacteria." (Ed): Schaad, N.W. 2nd ed. Am. Phytopathol. Soc. St. Paul, MN, USA, pp. 158.
13. Jones, A. L. 1971. Bacterial Canker of Sweet Cherry in Michigan. *Plant Dis.*, **55**: 961-965.
14. Jones, J. B., Dawe, D. L., and MacCurter, S. M. 1983. Separation of Isolates of *Pseudomonas syringae* pv. *Tomato* into Serovars by Three Serological Methods. *Phytopathology.*, **73**: 573-576.
15. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two Simple Media for the Demonstration of Pyocyanin and Fluorescin. *J. Lab. Clin. Med.*, **44**: 301-307.
16. Laemmli, V. K. 1970. Cleavage of Structural Proteins During Assembly of the Head of Bacteriophage T4. *Nature.*, **227**: 680-685.
17. Latorre, B. A., and Jones, A. L. 1979. *Pseudomonas morsprunorum*, the Cause of Bacterial Canker of Sour Cherry in Michigan and its Epiphytic Association with *Pseudomonas syringae*. *Phytopathology.*, **69**: 335-339.
18. Lovrekovich, L., Klement, Z., and Dowson, W. J. 1963. Serological Investigation of *P. syringae*, and *P. morsprunorum* Isolates. *Phytopathol. Zeitsch.*, **47**: 19-24.
19. Lucas, L.T., and Grogan, R.G. 1969. Some Properties of Specific Antigens of *Pseudomonas Lachrymans* and other *Pseudomonas* Nomenclatures. *Phytopathology.*, **59**: 1913-1917.
20. Maloy, S.R. 1990. Experimental Techniques in Bacterial Genetics. Jones & Baartlett Publ. 180 pp.
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y. 545. pp.



22. Okabe, N., and Goto, M. 1963. Bacteriophages of Plant Pathogens. *Annu. Rev. Phytopathol.*, **1**: 397-418.
23. Palleroni, N. J., Ballard, R. W., Rolstone, E., and Doudoroff, M. 1972. Deoxyribonucleic Acid Homology Among some *Pseudomonas* species. *J. Bacteriol.*, **110**: 1-11.
24. Palleroni, N. J. 1984. Family *Pseudomonaceae* Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith. 1917. In: "*Bergey's Manual of Systematic Bacteriology Vol. 1.*" (Eds): Krieg, N.R., and Holt, J.G., Williams and Wilkins Co., Baltimore, pp. 141-219.
25. Palmer, B.C., and Cameron, H.R. 1971. Comparison of Plant Pathogenic *Pseudomonas* by Disk Gel Electrophoresis. *Phytopathology.*, **61**: 984-986.
26. Rahimian, H. 1991. Bacterial Leaf Spot of Geranium in Mazandaran and Semnan Provinces. *Iran. J. Plant Pathol.*, **27**: 1-12.
27. Roos, I. M. M., and Hattingh, M. J. 1983. Fluorescent Pseudomonads Associated with Bacterial Canker of Stone Fruit in South Africa. *Plant Dis.*, **67**: 1267-1269.
28. Roos, I. M. M., and Hattingh, M. J. 1986. Bacterial Canker of Sweet Cherry in South Africa. *Phytophylactica.*, **18**: 1-4.
29. Roos, I. M. M., and Hattingh, M.J. 1987a. Systemic Invasion of Cherry Leaves and petioles by *Pseudomonas syringae* pv. *morsprunorum*. *Phytopathology.*, **77**: 1246-1252.
30. Roos, I. M. M., and Hattingh, M. J. 1987b. Pathogenicity and Numerical Analysis of Phenotypic Features of *Pseudomonas Syringae* Strains Isolated from Deciduous Fruit Trees. *Phytopathology.*, **77**: 900-908.
31. Rudolph, K. E. 1995. *Pseudomonas Syringae* Pathovars. In: "*Pathogenesis and Host Specificity in Plant Disease.*" (Ed): Singh, U.S., Elsevier Science, pp. 321.
32. Shamsbakhsh, M., and Rahimian, H. 1997. Comparison of Pathogenic Strains of *Pseudomonas syringae* on Various Plants in Iran. *Iran. J. Plant Pathol.*, **33**: 133-143.
33. Sundin, G.W., Jones, A. L., and Fulbright, D. W. 1989. Copper Resistance in *Pseudomonas Syringae* pv. *syringae* from Cherry Orchards and Its Associated Transfer *in Vitro* and *in Planta* with a Plasmid. *Phytopathology.*, **79**: 861-865.
34. Xu, G. W., and Gross, D.C. 1988. Physical and Functional Analysis of *Syr A* and *Syr B* Genes Involved in Syringomycin Production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.*, **170**: 5680-5689.
35. Young, J. M. 1991. Pathogenicity and Identification of the Lilac Pathogen, *Pseudomonas syringae* pv. *Syringae* Van Hall 1902. *Ann. Appl. Biol.*, **118**: 283-298.
36. Young, J. M., and Triggs, C. M. 1994. Evaluation of Determinative Tests for Pathovars of *Pseudomonas syringae* Van Hall 1902. *J. Appl. Bacteriol.*, **17**: 195-207.

خصوصیات فنوتیپی سویه‌های ایرانی *Pseudomonas syringae* pv. *syringae* van Hall بیماری شاتکر باکتریایی درختان میوه هسته دار

چکیده

در این تحقیق ۲۷ سویه در بین سالهای ۷۶-۱۳۷۴ از شانکرهای درختان میوه هسته‌دار شامل زردآلو، گیلاس، هلو، آلو، قیسی و شلیل از مناطق اطراف کرج جدا شد. براساس آزمونهای گروه LPOAT و GATTA's عامل بیماری *Pseudomonas syringae* pv. *syringae* شناسایی گردید. سویه‌ها براساس خصوصیات مرفولوژیکی و فنوتیپی (بیوشیمیایی و فیزیولوژیکی)، سرولوژیکی، پروفیل DNA پلازمیدی، نقوش پروتئین محلول سلولی (SDS-PAGE) و آنتی بیوگرام اختلاف بسیار جزئی نشان دادند. قدرت بیماریزایی سویه‌های تحت بررسی براساس میزان تولید سرینگومایسین در شرایط *in-vitro* و ایجاد ناحیه

تکروزه روی میوه نارس گیلاس اندازه گیری شد و همبستگی بالایی نشان داده و بین سویه‌ها از نظر قدرت بیماریزایی اختلاف معنی داری وجود داشت.