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Comparisons of Iranian Strains of *Pseudomonas syringae* pv. Syringae from Various Hosts with Different Methods

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ABSTRACT

A total of 46 strains of *Pseudomonas syringae* pv. syringae (Pss), including four reference strains, from different hosts and various geographical regions of Iran were compared based on their phenotypic, biochemical, serological and total protein properties. Pss strains showed similar phenotypical reactions except for aesculin hydrolysis, ice nucleation activity, syringomycin production, tyrosinase activity and utilization of adonitol, L-cysteine, formate, L-proline, raffinose and tartarate. All strains possessed a similar antibiogram profile towards 28 antibiotics tested. The strains were divided into three groups of syringomycin using *Geotrichum candidum* in standard bioassay. Three strains were used in antisera preparation against Pss in rabbit for serological studies. Ouchterlony double diffusion test divided Pss strains into six serological groups, when the results of heat-treated and non-treated whole bacterial cells used were combined for analysis and into seven serological groups when heat-treated antigens were used. All three antisera were able to identify Pss strains from various hosts in dot-immunobinding assay. This technique detected as few as 10^5 Pss cells per 10 µL reaction mixture from a pure culture. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed a significant homology in soluble protein patterns among Pss strains from stone fruits, barley and wheat except for those from sugarcane. Additional protein bands were found in strains from sugarcane. Results suggested that Pss strains from various hosts and geographical regions of Iran have adapted themselves to specific niches and hosts, acting differently with respect to utilization of carbon sources, etc. and thus belong to distinct taxonomic groups.

Key Words: Biochemical; DIBA; Phenotypic; Serology; SDS-PAGE

INTRODUCTION

Pseudomonas syringae pv. syringae van Hall 1902 (Pss) is the causal agent of many plant diseases including bacterial canker of stone fruits, red streak of sugarcane, blight of wheat and barley, brown spot of bean, citrus blast, bacterial spot of sorghum and maize holcus spot (González, 2003). Bacterial canker of stone fruits, also known as twig blight, blossom blight, gummosis, die back and spur blight, is one of the most devastating diseases of stone fruits that results in yield reduction between 10-20% in young orchards and even up to 80% under favorable climatic conditions (Young, 1991). The pathogen attacks twigs, buds, flowers, leaves and fruits. In the early spring, dark brown sunken lesions appear on twigs underneath the infected spurs. Severe infection of twigs results in shoot blight and death of infected branches with gums often appearing from cankered regions on the limbs. Red streak of sugarcane is another bacterial disease caused by Pss in which the photosynthetic rate and so the yield tend to decline. Pss can also cause small pale green water soaked lesion on barley and wheat leaves. Under favorable climatic conditions the disease rapidly spreads to flag leaf during heading, blighted spots coalesce, become irregular and turn yellow to brown. Despite infecting a wide variety of plants from different genera, bacterial host range is not determined or a single strain is capable of infecting a large number of plants (Young, 1991).

P. syringae is a very heterogeneous group and belong to RNA homology group I of *Pseudomonas*, which is part of subclass γ from class proteobacteria (Young, 1991) suggested eleven biochemical tests for identification. He believes that (a) there are a few strains with little homology to Pss and may be classified as a new species, (b) other may be phenotypically similar to or different from Pss and their differences may lie in host range and (c) some strains have distinct phenotypic properties with 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 identification of Pss easier and much more reliable than conventional methods. BIOLOG system using 95 carbon sources has confirmed that Ps is heterogeneous and specific pathovars are differentiated based on a single metabolic

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pattern. However no correlation has been found between biochemical characteristics and pathogenicity. Various methods have been employed for characterization of *Pseudomonas*. These include fatty acid profile, antigenic specificity, phage typing and syringomycin production (Lucas & Grogan, 1969; Okabe & Goto, 1963). DNA-DNA homology is another method deciphering bacterial relationships but it suffers from defining pathogenicity. *P. syringae* pathovars exhibit DNA homology at 40-100%, whereas this between 95-100% among strains of one particular pathovar (Palleroni *et al.*, 1972). Iran is listed among the countries producing stone fruits, sugarcane, wheat and barley in the world (FAO). Bacterial canker of stone fruits, blight of cereals and red streak of sugarcane have previously been reported and characterized from Iran (Bahar *et al.*, 1985; Rahimian, 1995; Mohammadi *et al.*, 2001).

The purpose of this study was to compare Pss strains from a diverse group of hosts such as stone fruits, sugarcane, wheat and barley, bellis and herbiscus with respect to their phenotypical, serological and total protein properties.

MATERIALS AND METHODS

Bacterial sterains purification and preservation: The Pss strains used in this study are shown in Table I. Those included those isolated from stone fruits, cereals, sugarcane, bellis and hibiscus from four provinces in Iran with known Pathogenicity activity on their hosts and four reference strains from apricot for comparative purposes (Psallidas, Greece). Each strain was streaked on nutrient agar (NA) medium, single colonies were picked up and re-streaked on the same medium to ensure purity.

Phenotypic and antibiogram tests: Bacterial phenotypes were studied based on standard physiological and biochemical tests. These included gram stain using 3% (w/v) KOH according to Suslow (Klement *et al.*, 1990), oxidative/fermentative based on Hugh and Leifson (Hildebrand *et al.*, 1988), levan production following Lelliot and Stead (1987), fluorescent pigmentation on King's B medium (Hildebrand *et al.*, 1988) and oxidase (Kovacs, 1956), reducing compounds from sucrose (Dye), catalase, pectinase, gelatin hydrolysis, growth on 5 and 7% (w/v) NaCl, aesculin hydrolysis, starch hydrolysis, indole test, Tween-80 hydrolysis following Lelliot and Stead (1987), HR test on tobacco (Klement *et al.*, 1990), gas from glucose, nitrate reduction, urease (Hildebrand *et al.*, 1988), litmus milk on Difco medium. Arginine dihydrolase, H_2S production from cysteine, ice nucleation activity, *3-keto*-lactose and methyl red and acetoin (MR-VP), lecithinase, tyrosinase, casein hydrolysis and optimal growth temperature were carried out according to Fahy and Hayward (1983). Carbon utilization test was done by adding appropriate sugar, amino acid, organic acid, organic salt, etc., to basal medium. Results were evaluated for 30 days in all above experiments. Each carbon source was tendalized separately and then added to the basal medium at a final concentration of 0.5% (w/v). Syringomycin production bioassay was carried out in two method using *Geotrichum candidum* plug in the center of plate and spray of fungal spore suspension on surface of PDA plate (Young, 1991; Bultreys & Gheysen, 1999).

Antibiogram test was performed on nine representative strains of Pss using paper disks impregnated with different antibiotics. One hundred µL of bacterial suspension (1×10^9 cells mL^{-1}) was spread over NA medium supplemented with 1% (w v^{-1}) glucose in petriplate (Psallidas, 1993). Sterile antibiotic disks were then placed 2-3 cm from each other on the medium. The inhibition zone was measured after 24-48 h of incubation at 25°C. Each experiment was repeated twice.

Serological test: Polyclonal antisera against three strains from sugarcane, barley and sweet cherry were prepared according to Jones *et al.* (1983). Microagglutination test was done to determine antiserum titer according to Ball (1990). Phosphate buffer was used as a control. Droplets were examined under dark field microscope (Carl Zeiss-Germany) for the presence of precipitate. Ouchterlony double diffusion test was carried out according to Schaad *et al.* (2001). Autoclaved (121°C, 20 min) and non-autoclaved whole bacterial suspension cells were prepared at OD_{600} nm 1.5-2.0. The central well in agarose gel was charged with 25-30 µL of antiserum and the other wells with homologous Pss antigens from the corresponding hosts. Negative controls included *Agrobacterium vitis* and *Bacillus subtilis.* IgG were purified from the antisera by mixing 1 mL of antiserum with 9 mL deionized water according to Ball (1990). Dot-immunobinding assay (DIBA) was performed with slight modification from Klement *et al.* (1990). Pss homologues were prepared at various dilutions from 10 to 109 CFU mL^{-1} and used in evaluating the sensitivity of this method. Sterile water was used as a negative control. Ten µL of antigen was dotted on nitrocellulose membrane after which the blot was dried and then blocked in 5% (w/v) fat-free milk in Tris-buffered saline (TBS) for one h. The immunoblot was washed in TBS containing 0.05% (v/v) Tween20 (TTBS) and TBS twice each for 5 min and then incubated in anti-Pss polyclonal antiserum diluted from 1:1000 to 1:4000 in 2.5% (w/v) fat free milk as the first antibody for 2 h, and washing was done as given before. The immunoblot was incubated in goat-antirabbit AP conjugate prepared at 1:2000 in TBS containing 2.5% fat free milk as the second antibody for 2 h. The blot was soaked in developing solution until the dots appeared.

SDS-PAGE: SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out under denaturing condition as described by Laemmli (1970). The resolving gel was 12% and the stacking gel was 4%. Pss strains were grown up on NAG for 24 h. Bacterial cells suspended in sterile water with OD_{600} nm of 2 were centrifuged at 2576 g for 5 min and resuspended in 1 mL

distilled water. Sample buffer containing mercaptoethanol and SDS was added to each tube. Samples were heated in a boiling water bath for 5 min and centrifuged at 6800 g for 10 min. Forty five mL of the supernatant containing soluble proteins was loaded in each well. Electrophoresis was carried out at a constant voltage of 100 volts. Staining of the gel was done in Coommassie brilliant blue G250.

RESULTS

Results of phenotypic and biochemical tests for all Pss strains for Gram staining, oxidase, pectinase and arginine dihydrolase were negative. With the exception of cereals, all other strains produced levan polymer on NA supplemented with 5% glucose. Pss strains induced the HR on tobacco leaves and produced pyoverdin on King's B medium with a varying degree. Gelatin, tween80 and casein hydrolysis were positive but none of the strains were able to hydrolyze starch. Strains could not grow at 4 or 41°C. They were aerobic and catalase positive. Both tyrosinase activity and nitrate reduction were negative for all Pss strains studied. Except for a few strains isolated from sugarcane, cereal and bellis, all other strains exhibited ice nucleation activity. Litmus milk test was alkaline. Production of reducing compounds from sucrose, urease and growth on 5% (w/v) NaCI were positive, whereas none of the strains tolerated 7% (w/v) NaCl. Lecithinase was negative, but phosphatase was positive. In addition, H_2S formation from cysteine, gas from glucose, methyl red and acetoin (MR-VP), indole production, sodium tartarate and *3-keto*-lactose were negative for all Pss strains. With the exception of six strains, syringomycin production was positive with varying degrees of inhibition zone. Pss strains were able to utilize various sugars as a sole carbon source by changing the medium from green to yellow indicative of acid formation. A few strains representing different hosts behaved atypically with respect to aesculin hydrolysis, ice nucleation activity, syringomycin production, tyrosinase activity and utilization of adonitol, L-cysteine, fumarate, raffinose, tartarate and trehalose as shown in Table II. Syringomycin production was variable among Pss strains. A total of 29 strains (8 sugarcane, 13 stone fruits, 5 cereals, 2 bellis & 1 hebiscus) showed strong production of syringomycin, 11 strains were weak producers (4 sugarcane, 5 stone fruits & 2 cereals) and the remaining six produced none. Standard strain BPIC243 was placed in the first group, whereas the other three in the second group. There was no difference between fungal disk and spore spray method for fungal application on medium regarding growth inhibition of *Geotrichum candidum.* Antibiogram test was carried out using nine representative Pss strains together with four reference strains against 28 antibiotics. Based on the growth inhibition zone caused by antibiotics, strains fell into four groups. Group 1, resistant to all antibiotics; group 2, partially sensitive showing inhibition zone of 1-4 mm; group 3 sensitive showing inhibition zone of 5-10 mm and group 4, highly sensitive whose inhibition zone exceeded 10 mm in diameter. There were no significant variations among Pss strains from different hosts with respect to antibiotic sensitivity.

Table I: Some ecological details of Pss strains used in this study

Strain	Host plant	Locality
1-12	Sugarcane	Mazandaran
13	Apricot	Shahr-e-kord, Charmahal
14,15	Apricot	Shiraz, Fars
16	Apricot	Karaj, Tehran
17	Apricot	Mazandaran
18	Peach	Shiraz, Fars
19	Peach	Shahriar, Tehran
20	Peach	Baraghan, Tehran
21	Peach	Takestan, Qazvin
22	Peach	Mazandaran
23,24	Almond	Maharloo, Fars
25-28	Almond	Shahr-e-kord, Charmahal
29	Sweet cherry	Damavand, Tehran
30-32	Sweet cherry	Savojbalagh, Tehran
33,34	Wheat	Shahr-e-kord, Charmahal
35,36	Wheat	Shiraz, Fars
37	Barley	Shiraz, Fars
38	Barley	Karaj, Tehran
39	Barley	Shiraz, Fars
40	Barley	Mazandaran
41-43	Bellis	Mazandaran
44-46	Hibiscus	Mazandaran
BCIP219,BCIP224, BPIC242,BPIC243	Apricot	Reference strains*

*****Reference strains were gift by P.G. Psallidas, Benaki Phytopathological Institute Collection, Benaki. Greece

Table II: List of Pss strains showing atypical phenotypical and biochemical reactions

Characteristic	Reaction	Strains
Aesculin hydrolysis	-	14, 22, 30, 33, 35, 38
Ice nucleation activity	-	6, 33, 35, 37, 41
Syringomycin production	-	16, 30, 39, 41, 44, 45
Tyrosinase activity	+	4, 7, 9, 12, 13, 14, 19, 25, 30, 34, 36
Utilization of:		
Adonitol	+	8, 19, 27, 29, 35
L(-)Cysteine	+	7, 21, 27, 28, 36
Fumarate	+	6, 8, 19, 29, 38
Raffinose	+	3, 4, 7, 10, 12, 23, 32, 33, 34, 38, 39, 42, 46
Tartarate	+	14, 36
Terhalose	+	12, 22, 27, 34, 35

Micro-agglutination test using homologous antigens revealed antisera titers for sugarcane, barley and sweet cherry strains to be 1/1024, 1/1024 and 1/512, respectively. Phosphate buffer was used as a negative control in which there were no cross-reactions between various dilutions of antiserum and antigens. Ouchterlony double diffusion test was performed using intact as well as heat killed bacterial antigens against the three polyclonal antisera prepared from the corresponding sugarcane, barley and sweet cherry strains. As shown in Table III, Pss strains fell into six serological categories when the results of non-treated intact and heat killed bacterial antigens were combined. Some strains cross-reacted with their own homologous antiserum only whereas others showed positive reaction toward one or

Table III: Serological grouping of Pss strains based on combined results from non-treated intact and heat-treated antigens

Group	Antiserum	Total number of strains	Strain number
1	ABC	14	2, 3, 6, 11, 12, 16, 17, 18, 23, 24, 28, 30, 31, 34
2	A	5	1, 4, 5, 9, 10
3	B	5	33, 36, 37, 41, 43
4	C	10	13, 14 ,19, 20, 21, 25, 29, 32, 44, 46
5	AB	4	7, 8, 35, 38
6	BC	8	15, 22, 26, 27, 39, 40, 42, 45

A, Anti-sugarcane strain antiserum
B, Anti-barley strain antiserum
C, Anti-sweet cherry strain antiserum

Table IV: Serological grouping of Pss strains based on results from heat-treated antigen only

Group	Antiserum	Total number of strains	Strain number
1	ABC	6	3, 6, 18, 30, 31, 34
2	A	9	1, 2, 4, 7, 9, 10, 11, 12
3	B	6	33, 35, 37, 41, 45
4	C	14	13, 14 ,16, 17, 19, 20, 21,22, 23, 24, 25, 28, 29, 44
5	AB	2	8, 38
6	BC	3	15, 27, 39
7	-	6	27, 32, 36, 42, 43, 46

A, Anti-sugarcane strain antiserum
B, Anti-barley strain antiserum
C, Anti-sweet cherry strain antiserum

two heterologous antisera as well. In case when the results of heat-treated antigens were considered only, seven serological groups were emerged and greater number of strains cross-reacted with different antisera and thus acting more specifically with a fewer antisera (Table IV). A seventh group constituting a total of six strains did not cross-react with any of the antisera used. In dot-immunobinding assay, all three antisera were able to recognize and cross react with the representative strains of Pss from different hosts. Dot-immunobinding assay sensitivity for homologous antigens was tested using serial dilutions. The limit of detection for Pss strains from sugarcane and barley was 1,000 cells and for sweet cherry was 10,000 cells per 10 µL spot using the corresponding antisera, respectively.

SDS-PAGE analysis revealed a significant homology in banding pattern among Pss strains from stone fruits or sugarcane (data not shown). Further, total soluble protein profile from cereals, bellis and hebiscus showed considerable similarity to those representing stone fruits. There were, however some quantitative as well as qualitative variations in banding pattern among the two groups. These homologies were also noticeable with the four standard strains used in this study. On the other hand, we observed that sugarcane strains exhibited distinct banding pattern as compared to Pss strains from other groups.

DISCUSION

The *P. syringae* pv. syringae (Pss) is one of the 57 pathovars that infects as many as 180 plant species belonging to several unique families (Ramos *et al.,* 2007). In this study, a total of 46 Pss strains were identified based on LOPAT (levan, oxidase, potato rot, arginine dihydrolase & HR on tobacco), nitrate reduction, syringomycin production and ice nucleation activity tests. All together, a total of 87 phenotypic and biochemical tests were performed on these strains for further characterization.

Results from physiological and biochemical tests were consistent with those previously reported for Pss (Rahimian, 1995; Mohammadi *et al.*, 2001). In some phenotypic tests such as aesculin hydrolysis, syringomycin production, ice nucleation activity, tyrosinase activity and utilization of adonitol, L-cysteine, fumarate, raffinose, tartarate and trehalose, Pss strains from Iran behaved differently from those reported from elsewhere (Table II). Young (1991) suggested that such variations more likely reflect bacterial adaptation to the corresponding host plants and the use of different food sources. All Pss strains were HR positive. Previously we reported some degree of host specificity among Pss strains isolated from stone fruits and wheat and barley (Mohammadi *et al.,* 2002). According to Little *et al.* (1998), the susceptible host for the pathovar syringae is peach var. Lovell though it is not its main host. Further evidence also shows host specificity among Pss strains.

Pss strains in this study varied in regard to the degree of *in vitro* syringomycin production. Previous study indicated that syringomycin production was correlated with degree of pathogenicity on immature sweet cherry fruits. In some situations, phytotoxin production within a pathovar has been correlated with pathogenicity on specific hosts (Mohammadi *et al.*, 2001). It is noteworthy that as a result of culturing the pathogen on artificial media, some phenotypic characteristics including syringomycin production may increase or decrease (Young, 1991). Further, it has been shown that a particular strain may produce syringomycin *in vitro,* yet it may be nonpathogenic on the host plant. This has been demonstrated with Pss strains from wheat, sour cherry and pea. Antibiogram test was performed with disks on nine representative strains of Pss and the inhibition zone was measured after 24-48 h. Different strains showed very high sensitivity for tetracycline, oxytetracycline, and high sensitivity for nalidixic acid, tobramycin and streptomycin. Results from Antibiogram test were consistent with those previously reported for Pss (Mohammadi *et al.*, 2001).

In serological experiments three polyclonal antisera against Pss strains sugarcane (#5), barley (#37) and sweet cherry (#29) were used. In ouchterlony double diffusion test Pss strains fell in six serological groups when the results of non-treated intact and heat-killed bacterial antigens were combined. They fell in seven serological groups, when the results of heat-treated antigens were considered. In fact, the reaction in second case was more specific and Pss strains

reacted with less number of antisera. Six strains from various hosts did not cross-react with any of used antisea and fell in distinct group. In line with these dete, Mazarei *et al.* (1992) in a similar study on Pss and *P. syringae* pv. pisi used heat-killed bacteria as antigen and recognized these pathovars as distinct categories. Lucas and Grogan (1969) demonstrated all plant pathogenic *Pseudomonas* have several antigen in common and each species has a specific antigen that will not be remove with heat. Lovrekovich *et al.* (1963) provided some evidence of specific reaction of heat-resistant antigen. In Dot immunobinding assay, contrary to our expectation, no high sensitivity was observed. In this assay three antisera had positive reactions with representative Pss strains. Although most reaction was for homologous host strain, there was somehow the same reaction with another host strains. Due to high sensitivity of this technique, anti-barley and anti-sugarcane antisera were detected up to 10^7 cell mL^{-1} and for sweet cherry antiserum was up to 10^8 cell mL^{-1}. These were not good results. Serological tests with polyclonal antibody needed fewer facilities and were cost-effective. However for better test results, it is better to use monoclonal antibody for each strain, which gives exact results.

SDS-PAGE analysis of total soluble protein profile revealed strong homology in banding patterns within and among strains of stone fruits, wheat, barley, bellis and hibiscus. Sugarcane strains were somewhat different from those isolated from stone fruits since they possessed a few additional protein bands of 30-45 kDa. These results were found consistent with those reported by Rahimian (1995). Other studies have shown lack of variations in protein pattern among Pss from different hosts. Such differences were reported to exist between Pss and *P. syringae* pv. pisi or other pathovars (tabaci, savastanoi & mellea).

Overall, based on the above results, Pss strains from various hosts and geographical regions seemed to be different based on phenotypical, serological and molecular properties. It is likely that Pss strains from various hosts may have adapted themselves to specific niches and hosts acting differently with respect to utilization of carbon sources, etc. and thus may belong to distinct taxonomic groups.

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