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Comparison of *P. syringae* pv. *syringae* from Different Hosts Based on Pathogenicity and BOX-PCR in Iran

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ABSTRACT

During 2007-2008, 58 strains of *P. syringae* pv. *syringae* (*Pss*) were isolated from various *Prunus* species and other hosts such as sugar beet, pear, quince, oat, millet, wheat, barley, and rice in Fars, Isfahan, Kohgiloye and Boyer Ahmad, Chahar Mahal-o-Bakhtiari **provinces of Iran. The strains were tested for pathogenicity, the presence of the *syrB* gene and BOX PCR (BOX A1R primer). All tested *Pss* strains were pathogenic on peach seedlings regardless of their original hosts. A total of 58 isolates of the *Pss* and *Pss* IVIA 773-1 amplified a 752-bp fragment with the *syrB* primers. The results of analysis of the BOX fingerprints from *P. syringae* pv. *syringae* strains showed that the strains isolated from stone fruits, graminous hosts and pome fruits formed a relatively distinct cluster, which were separable from the strains isolated from the other hosts. Results of this study indicate the existence of a relative degree of host specialization within the heterogeneous pathovar *Pss*.**

Keywords: BOX-PCR, Pathogenicity, *P. syringae* pv. *syringae*, Stone fruit, *syrB* gene .

INTRODUCTION

P. syringae pv. *syringae* (*Pss*), the causal agent of bacterial canker and blast of stone fruit trees, is one of the most important plant pathogen in the world. *Pss* is a particular bacterium among *P. syringae* pathovars due to its capacity to cause disease in many species of plants (Little *et al*., 1998). Traditionally, strains of *Pss* are recognized based on biochemical, nutritional, and physiological characteristics and ability of pathogenicity on lilac and peach seedling (Little *et al.,* 1998; Scortichini *et al*., 2003; Vicente and Roberts, 2003; Vicente and Roberts, 2007; Gilbert *et al*., 2009; 2010). *P. syringae* strains that are found infecting a host and are similar to *Pss* strains based on biochemical and nutritional characteristics have been assigned in this pathovar. In many cases, biochemical and nutritional tests are not the best methods to differentiate strains at or below the pathovar level and pathogenicity test in greenhouse is not a suitable index of natural host preference (Little *et al*., 1998). The analysis of DNA based on nucleic acid hybridization revealed that *P. syringae* is a heterogeneous species (Pecknold and Grogan, 1973). Nine genomic species were described within *P. syringae* on the basis of the results of DNA studies. Strains belonging to several pathovars of *P. syringae* include *P. s*. pv. *syringae*, *P. s*. pv. *aptata, P. s*. pv. *pisi, P. s*. pv. *papulans* were clustered in genomic species I (Gardan *et al*., 1999). The *Pss* host specificity among the strains that infect different hosts such as beans, grasses, and prunus species were reported on the basis of pathogenicity tests (Little *et al*., 1998). Many researchers have found that peach seedlings are sensitive to *Pss* strains from different hosts (Otta and English, 1971; Vicente and Roberts, 2007; Gilbert *et al*., 2010). Similarly, Lai and Hass (1973) showed that cowpea leaves have different

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susceptibility to *Pss* strains isolated from different hosts.

Genomic fingerprinting methods based on the polymerase chain reaction (PCR) have been applied for identification and classification of plant associated bacteria to the subspecies level (Louws *et al*., 1995 and 1999). Rep-PCR technique is a useful method to differentiate and classify bacterial strains below the level of species (Versalovic *et al*., 1991). Rep-PCR is based on DNA primers corresponding to naturally occurring repetitive elements in bacteria, such as the REP, ERIC and BOX elements (Versalovic *et al.,* 1994). In this method, the prior knowledge of target DNA sequence is not necessary (Louws *et al*., 1999). Several *Pseudomonas syringae* pathovars including *phaseolicola, glycinae, tabaci, lachrymans* and *mori* that were studied based on ERIC- and REP-PCR showed that, in many cases, differences among strains within a pathovar were small. The researchers concluded that these methods could be used to identify and classify strains of the *Pseudomonas syringae* pathovars (Weingart and Volksch, 1997).The Australian isolates of *Pseudomonas syringae* pv. *pisi* and *Pss* were compared with rep-PCR. Using DNA fingerprinting, it was possible to distinguish these two pathovars and races 2 and 6 of *Pseudomonas syringae* pv. *pisi* (Hollaway *et al*., 1997). Rep-PCR methods, particularly BOX-PCR, proved to be useful for identifying the *Psm* race 1 and *Psm* race 2 isolates (Gilbert *et al*., 2009). In the same study, combined genetic results using rep-PCR and IS50-PCR confirmed high diversity in the pv. s*yringae*, in which homogeneous genetic groups were found on the same hosts (pear, cherry, and plum). Analysis of the ERIC fingerprints of *Pss* strains isolated from stone fruits in California showed that these strains formed a distinct cluster that could be separated from the strains isolated from the other hosts (Little *et al*., 1998). Another study revealed that different strains within *P.s. pv. pisi* could be separated in two distinct groups using Rep-PCR method (Suzuki *et al*., 2003).

Analysis of *Pss* and *P. syringae* pv. *morsprunorum,* isolated from cherry in UK using rep-PCR, showed that this method can easily distinguish these two major pathovars (Vicente and Roberts, 2007). Moreover, researchers found that nine genomic groups, proposed by Gardan *et al*. (1999) could be distinguished using BOX- PCR. They showed that the results corresponded with Gardan's results (Marques *et al*., 2008).

In Iran, *Pss* strains were isolated from various plants of different areas and characterized (Bahar *et al*., 1982; Bana pour *et al*., 1990; Al-e-Yasine and Banihashemi, 1993; Elahi nia and Rahimian, 1996; Afionian *et al*., 1996; Afionian and Sahragard, 1996; Shams bakhsh and Rahimian, 1997; Ghasemi *et al*., 1998; Mohammadi *et al*., 2001; Taghavi and Ziaee, 2003; Ashorpour *et al*., 2008). In many cases, they emphasized phenotypic and nutritional characteristics of the pathogen and showed differences between isolates from various hosts, whereas genotypic features of this important plant pathogen have not been studied yet. Recently, using ERIC- and BOX-PCR primers, it was shown that the fingerprints of the strains isolated from sugarcane were distinct from those of the strains isolated from stone fruits and wheat. The results indicated that the *Pss* strains isolated from sugarcane with red streak symptom constitute a group genotypically distinct from those inciting canker on stone fruit trees and blight of wheat (Mosivand *et al*., 2009).

The aim of this study was to compare and differentiate strains of *Pss* isolated from various *Prunus* species and other hosts such as sugar beet, pear, quince, oat, millet, wheat, barley, and rice by using pathogenicity test and BOX-PCR analyses.

MATERIALS AND METHODS

Isolation

During 2007 to 2008, samples of both healthy and diseased tissues of stone fruit trees such as apricot, peach, cherry, almond, wild almond, sugar beet, pear, quince, oat, millet, wheat, barley, rice, rose, pelargonium

and *Malva* sp were collected from different orchards in Fars, Isfahan, Kohgiloye and Boyer Ahmad, Chahar Mahal-o-Bakhtiari provinces of Iran. The tissues were surface sterilized in 1% sodium hypochlorite for 1 minute, rinsed in sterile water, ground in a small amount of phosphate buffer (PB) and 2 ml of liquid suspension was spread on King`s B medium. After incubation for 4 days, the fluorescent colonies were purified and tested for LOPAT tests (oxidase reaction, the ability to rot potato slices, presence of arginine dihydrolase, levan production, and tobacco hypersensitivity) (Lelliot *et al*., 1966; Schaad *et al*., 2001). Characteristics of bacterial isolates used in this study are listed in Table 1. The *P*ss (IVIA773-1) and *P. savastanoi* pv. *savastanoi* (IVIA 2558-IT), from Instituto Valenciano de Investigaciones Agrarias (IVIA) Spain, were used as reference strains in this study.

Pathogenicity Test

Bacterial strains were grown for 48 hours on KB medium at 25°C and were suspended in Phosphate Buffer (PB) to a concentration of 10^7 CFU (OD600= 1). One ml of bacterial suspensions was injected into the green stems of peach seedlings by using a needle (Little *et al*., 1998). Each plant was inoculated in five places with one strain and was covered with parafilm at the injection site. One isolate of *P. savastanoi* pv. *savastanoi*, was also injected into the stem of peach seedling. PB was injected as a control to peach seedlings. Peach seedlings were maintained in a greenhouse at 28°C and rated after 2 weeks for symptoms development.

DNA Preparation

All strains were grown on KB medium at 25°C for 3 days. A loopful of colony from each strain was suspended in sterile distilled water to a concentration of 10^7 CFU (OD600= 1). The suspensions were boiled for 8-10 minutes and after cooling in the room temperature, were used as template DNA for pathovar-specific PCR and BOX-PCR (Clerc *et al*., 1998).

Identification of Pss with Specific Primers

Two 21-mer oligonucleotides from *syr B* gene [primer B1 (5'-CTTTCCGTGGTCTTGATGAGG-3') and primer B2 (5'-TCGATTTTGCCGTGATGAGTC-3')] were selected for PCR and were purchased from Metabion Co., Germany. The primers B1 and B2 locate into the open reading frame of the *syrB* gene and yield a 752-bp product (Sorensen *et al.,* 1998). The PCR reactions were performed in Bio-Rad I-cycler (USA) in 26 µl PCR mixture: 2 µl of DNA template was transferred to 24 µl of a PCR mixture containing 50 pmol of each primer, 0.2 mM dNTP mix, 2 U of *Taq* DNA polymerase (Metabion Co., Germany), and 1.6 mM magnesium chloride. The PCR reaction was carried out for 35 cycles using the following procedure: template denaturation at 94°C for 1.5 minutes, primer annealing at 60°C for 1.5 minutes, DNA extension for 3.0 minutes at 72°C and final extension at 72°C for 10 minutes. The PCR products were electrophoresed on 1 % TBE agarose gel at room temperature at 90 V cm^{-1} for 1 hour. Following staining with ethidium bromide, the gels were viewed and photographed under UV illumination.

BOX-PCR Conditions

The BOX-PCR was carried out with BOX A1R primer (Versalovic *et al*., 1991). BOX A1R primer [5'-CTACggCAAggCgACgCTgACg-3']) was purchased from Metabion Co., Germany. The PCR reactions were performed in

Table 1. Characteristics of bacterial strains used in this study.

Strain	Host	Location
P. syringae pv. *syringae*		
1.	Almond	Fars
2.	Barley	Fars
3.	Rice	Fars
4.	Oat	Kohgiloye and Boyer Ahmad
5.	Peach	Fars
6.	Rose	Fars
7.	Cherry	Fars
8.	Healthy Peach	Chahar Mahal-o-Bakhtiari
9.	Peach	Isfahan
10.	Almond	Kohgiloye and Boyer Ahmad
11.	Rose	Fars
12.	Unknown	*P. savastanoi* pv. *savastanoi* IVIA 2558-IT
13.	Apricot	Kohgiloye and Boyer Ahmad
14.	Peach	Fars
15.	Cherry	Chahar Mahal-o-Bakhtiari
16.	Beet	Fars
17.	Apricot	Fars
18.	Cherry	Fars
19.	Peach	Chahar Mahal-o-Bakhtiari
20.	Cherry	Fars
21.	Peach	Chahar Mahal-o-Bakhtiari
22.	Pelargonium	Fars
23.	Pear	Fars
24.	Peach	Isfahan
25.	*Malva*	Fars
26.	Wild Almond	Fars
27.	Peach	Isfahan
28.	Almond	Kohgiloye and Boyer Ahmad
29.	Wheat	Chahar Mahal-o-Bakhtiari
30.	Wild Almond	Fars
31.	Almond	Kohgiloye and Boyer Ahmad
32.	Peach	Chahar Mahal-o-Bakhtiari
33.	Peach	Isfahan
34.	Almond	Kohgiloye and Boyer Ahmad
35.	Peach	Fars
36.	Quince	Fars
37.	Peach	Chahar Mahal-o-Bakhtiari
38.	Peach	Isfahan
39.	Wild Almond	Fars
40.	Cherry	Chahar Mahal-o-Bakhtiari
41.	Peach	Fars
42.	Wheat	Chahar Mahal-o-Bakhtiari
43.	Wild Almond	Fars
44.	Almond	Kohgiloye and Boyer Ahmad
45.	Cherry	Fars
46.	Apricot	Kohgiloye and Boyer Ahmad
47.	Almond	Fars
48.	Pelargonium	Fars
49.	Pear	Fars
50.	Almond	Chahar Mahal-o-Bakhtiari
51.	Apricot	Fars

Table 1. continued

Strain	Host	Location
P. syringae pv. *Syringae*		
52.	Wild Almond	Fars
53.	Cherry	Chahar Mahal- o –Bakhtiari
54.	Cherry	Fars
55.	Wheat	Fars
56.	Unknown	*Pss*IVIA 773-1(Standard isolate)
57.	Wild Almond	Fars
58.	Cherry	Fars
59.	Almond	Isfahan
60.	Millet	Fars

Bio-Rad I-cycler (USA) in 26 µl PCR mixture: 2 µl of DNA template was transferred to 24 µl of a PCR mixture containing 45 pmol BOX A1R primer, 0.2 mM dNTP mix, 2 U of *Taq* DNA polymerase, 1.6 mM magnesium chloride. PCR was performed under the following conditions: 1 cycle at 95°C for 2 minutes; 35 cycles at 94°C for 1 minute, 52°C for 1 minute, and 65°C for 8 minutes; and a final extension cycle at 68°C for 16 minutes (Opgenorth *et al*., 1996; Versalovic, *et al.,* 1991). The PCR products were electrophoresed on 1 % TBE agarose gel at room temperature at 80 V cm^{-1} for 3 hours. The DNA fragments were visualized by staining with ethidium bromide and photographed under UV illumination.

Data Analysis

The amplified fragments of each strain were detected, using Total Lab (v.1.1) program and were scored as 1 (present) or 0 (absent) and pair wise comparisons were made of each unique pattern by using the SM similarity coefficient of the NTSYSpc Software (Exeter Software, New York) (Rademaker *et al*., 1998). A similarity matrix was generated by using the unweighted pair-group method (UPGMA) with averages. Phenograms were constructed with the Tree Display Option (Rolph, 2000).

RESULTS

Fifty-eight strains of *Pss* were isolated from almond, wild almond, peach, apricot, cherry, beet, pear, quince, oat, millet, wheat, barley , rice, pelargonium, *Malva* sp. and rose in Fars, Isfahan, Kohgiloye and Boyer Ahmad and Chahar Mahal-e–Bakhtiari provinces. The bacterium was detected in diseased samples and, as an epiphyte, on several apparently healthy plants. All *P. syringae* pv. *syringae* strains used in this study were negative for oxidase, potato rot, and arginine dihydrolase, but, positive for levan production and the hypersensitive response on tobacco.

Pathogenicity Tests

Twenty five strains of *Pss* isolated from different plants and one strain of *P. savastanoi* pv. *savastanoi*, were tested for pathogenicity on peach seedlings**.** All of the *Pss* strains were pathogenic on peach and produced progressive necrotic symptom on the inoculated site of the stem (Figure 1), but, *P. savastanoi* pv. *savastanoi* isolate was not.

Identification of Pss with Specific Primer

A total of 60 strains, including 58 strains of *Pss*, *Pss* IVIA 773-1 and *P. savastanoi*

Figure 1. Necrotic symptom on stem of peach seedling inoculated with *Pss* strain.

pv. *savastanoi* (IVIA 2558-IT), were tested for the presence of the *syrB* gene. All 58 isolates of the *Pss* and *Pss* IVIA 773-1, amplified a 752-bp fragment with the *syrB* primers as expected (Figure 2), whereas *P. savastanoi* pv. *savastanoi* did not.

BOX Analysis

The DNA fingerprints of 59 strains of *Pss* from different hosts (Table 1) were determined by BOX-PCR. Genomic fingerprints were generated for the isolates. More than 16 DNA fragments, ranging from 200 to 2500 bp in size, were amplified with BOX-PCR primer. The fingerprint patterns of strains of *Pss* are shown in Figure 3. The occurrence of a particular BOX fingerprint pattern clearly differentiated strains isolated form different hosts. Based on genomic fingerprints using BOX A1R primer, the strains formed three clusters. Cluster one contained the strains of stone fruit, healthy peach, *Malva sp.*, two strains of rose, and one strain from pelargonium. The second cluster contained the strains isolated from quince and pear, one isolate from pelargonium, two isolates from peach, and the standard isolate (*Pss* IVIA 773-1). The third cluster consisted of graminous strains, one peach isolate and one isolate from beet.

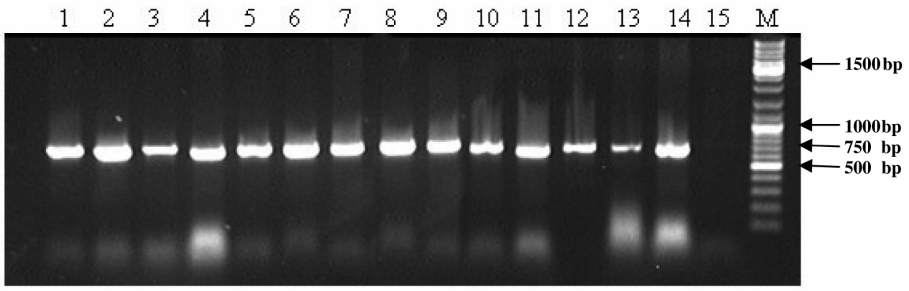

Figure 2. Agarose gel electrophoresis of PCR-products of *P*ss strains with primers B1 and B2. Left to right: 1-14: *Pss* strains; 15: Negative control, M: 100bp DNA molecular marker.

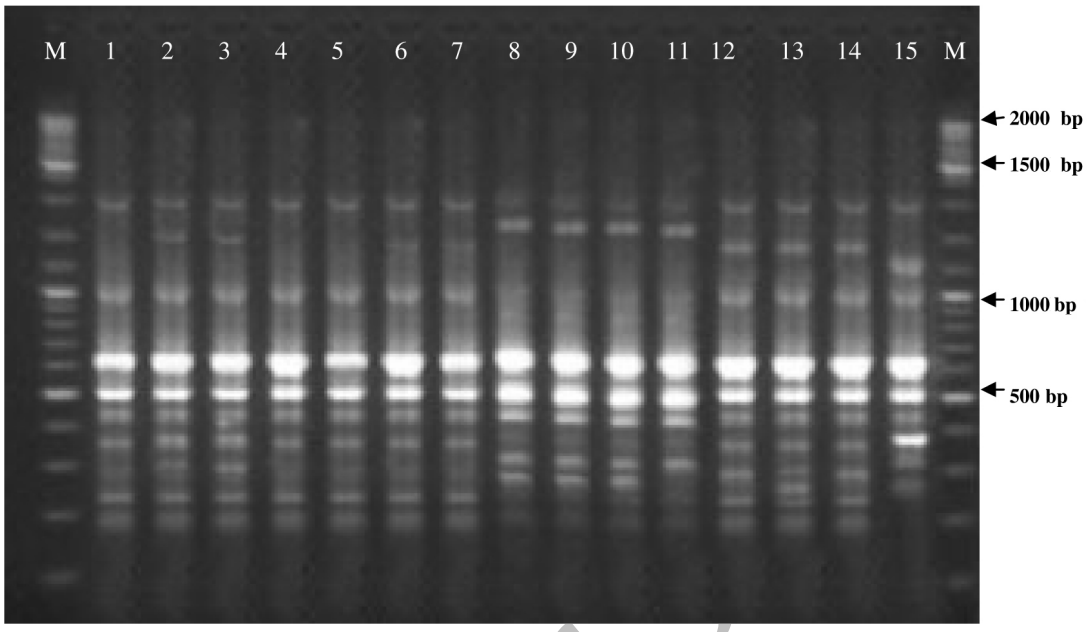

Figure 3. BOX-PCR fingerprint patterns of *Pss* strains isolated from different hosts. M: 100bp DNA molecular marker; 1: Healthy peach isolate; 2: Rose isolate; 3: Apricot isolate; 4: Cherry isolate; 5: Pelargonium isolate; 6: Wild almond isolate; 7: Almond isolate; 8: Wheat isolate; 9: Beet isolate; 10: Oat isolate; 11: Millet isolate; 12: Standard isolate (*Pss* IVIA 773-1); 13: Quince isolate; 14: Pear isolate, 15: *P. savastanoi* (IVIA2558IT).

Furthermore, *P. savastanoi* strain fell into a distinct cluster.

DISCUSSION

A total of 59 strains, including 58 strains of *Pss* and *Pss* IVIA 773 -1, amplified a 752-bp fragment with the *syrB* primers, whereas *P. savasatnoi* pv. *savastanoi* did not. These results show that all of the isolates could synthesize syringomycine, which is very important toxin for pathogenicity induction in plant. *P. s.* pv. *savastanoi*, that can not produce this toxin, didn't amplify the *syrB* gene.

In this study, the *Pss* strains isolated from various hosts in Fars, Isfahan, Kohgiloye and Boyer Ahmad, and Chahar Mahal-e-Bakhtiari provinces generated several genetic profiles in BOX-PCR. For example, almost all prunus isolates produced similar patterns. Similarly, graminous isolates and pome fruit isolates produced special patterns, which could be used for their differentiation from each other. The resulting dendrogram suggests a host specialization of several *Pss* strains within the heterogeneous pathovar *syringae*. The host specialization of *Pss* strains in a special host has been reported in prior studies. For example, bean pod pathogenicity assay have revealed that strains of *Pss* isolated from beans caused pathogenic reaction on been pods, whereas strains isolated from other hosts did not show this reaction (Saad and Hagedorn, 1972). Similar results have been reported in other studies of the strains isolated from beans (Cheng *et al*.,1989; Ercolani *et al*., 1974; Rudolph, 1979) and, therefore, a new pathovar has been proposed for bean strains as *P. syringae* pv. *phaseolicola* (Rudolph, 1979). Grass strains of *Pss* have been reported to be more virulent on inoculated maize plants than strains isolated from non grass hosts (Gross and DeVay, 1977). Moreover, analysis of 13 *Pss* strains isolated from sugarcane, wheat

and stone fruit, using ERIC- and BOX-PCR, showed that the strains that were isolated from sugarcane were distinct from those isolated from other hosts. Their results indicated that the *Pss* strains isolated from sugarcane constituted a genotypically distinct group from those pathogenic on stone fruit and wheat (Mosivand *et al*., 2009). In our study, the resulting dendrogram (Figure 4) suggests that the genomic fingerprints of *Pss* strains from stone fruits, pome fruit, and graminous hosts had more similarities to each other than to the other hosts. These results correspond with previous studies in Iran (Mosivand *et al*., 2009).

Our results indicate that pathogenicity test on peach seedlings can not distinguish *Pss* strains that are isolated from different hosts. These are similar to the results of other researchers who found that *Pss* strains from different hosts induced similar symptoms on peach seedling stems (Otta and English, 1971; Little *et al*., 1998).

Some weed plants within orchards have been supposed to serve as inoculum source for pathogen overwintering and disease development (Ercolani *et al*., 1974; Latorre *et al*., 1979; Roos and Hattingh, 1986). In this study, the BOX patterns of *Pss* strain isolated from *Malva sp*., which grows in orchards, was nearly similar to that of other strains that are virulent on prunus hosts**.** Moreover, pathogenicity test of this isolate on peach seedlings produced typical symptoms of *Pss***.** Accordingly, epiphytic *Pss* on weeds might have a role in the *Pss* overwintering. Epiphytic strains from healthy tissue generated the same banding patterns as the strains isolated from diseased tissues. Therefore, it seems that healthy tissues harbor populations of epiphytic strains of *Pss,* that are capable of causing

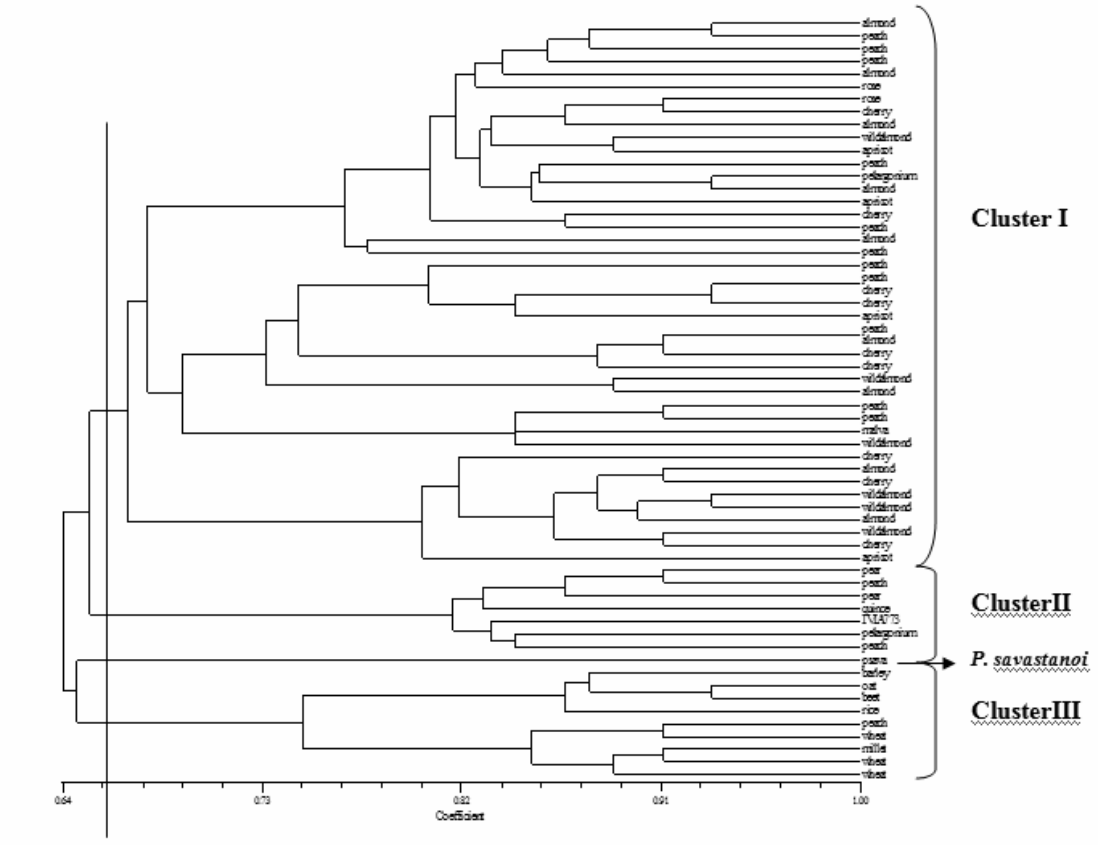

Figure 4. Dendrogram based on BOX-PCR of *Pss* isolates from different hosts.

bacterial canker in susceptible tissues. These results are in agreement with previous studies (Little *et al*., 1998).

Louws *et al*. (1995) found that rep-PCR methods are low cost, rapid, and reliable procedures to discriminate plant-pathogenic bacteria at the pathovar level . In this study, the results of comparison of *Pss* strains isolated from stone fruits, graminous plants and pome fruits showed that BOX-PCR can differentiate those isolates from each other. This result supports the hypothesis that, within the heterogeneous pathovar *syringae*, the strains infecting stone fruit, pome fruit, and graminous plant have adapted genetically to a particular host. Previously, a close relationship was reported between strains that infect pome fruits, such as pear, and stone fruits (Gross and DeVay, 1977; Roos and Hattingh, 1987).These findings are similar to the results of our study. REP-PCR analysis of 100 *Pss* strains from pear trees together with six strains from other hosts such as peach, wheat, tomato, and maize, showed that all of the pear strains clustered into one of the two nearly related groups, while the strains from other hosts did not have any similarities to the pear strains or to each other (Sundin**,** *et al*., 1994). On the other hand, few similarities were found in the ERIC patterns of five strains of *Pss* isolated from pear, apple, and cherry trees in Germany (Weingart and Völksch, 1997).

Our results suggest that strains of *Pss*, at least those isolated from stone fruits, graminous hosts, and pome fruit were relatively adapted to a special host. Likely, this adaptation resulted from a long lasting life of a population of genetically heterogeneous *Pss* strains on a special host.

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مقایسه جدایه‌های *P. syringae* pv. *syringae* از میزبان‌های مختلف، بر اساس آزمون بیماری‌زایی و BOX-PCR در ایران

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چکیده

طی سال های ۱۳۸۶و۱۳۸۷، پنجاه و هشت جدایه *Pseudomonas syringae* pv. *syringae* (*Pss*) از میزبان‌های مختلف، نظیر هسته‌داران، چغندرقند، گلابی، به، یولاف، ارزن، برنج، گندم و جو در استان‌های فارس، کهکیلویه و بویراحمد ، چهارمحال و بختیاری و اصفهان جداسازی گردید. جدایه‌ها بر اساس آزمون بیمای‌زایی، وجود ژن syrB و BOX-PCR مورد ارزیابی قرار گرفتند. کلیه جدایه ها، صرفنظر از نوع میزبان روی نهال‌های هلو بیماریزا بودند. تعداد ۵۸ جدایه *Pss* به همراه جدایه *Pss* 773-1 IVIA ، قطعه ۷۵۲ جفت بازی با آغازگر *syr*B را تکثیر نمودند. تجزیه و تحلیل انگشت نگاری ژنتیکی جدایه های *Pss* با استفاده از آغازگر BOXA1R نشان داد که جدایه‌های هسته‌داران، غلات و دانه‌داران گروه‌های نسبتاً متمایزی را تشکیل داده و از سایرین قابل تفکیک می‌باشند. این نتایج نشان‌دهنده وجود ترجیح میزبانی نسبی در میان جدایه‌های مختلف *Pss* می باشد.