CHARACTERIZATION OF *PSEUDOMONAS SAVASTANOI* pv. *SAVASTANOI* STRAINS ISOLATED FROM SEVERAL HOST PLANTS IN TURKEY AND REPORT OF FONTANESIA AS A NEW HOST

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SUMMARY

Several strains of *Pseudomonas savastanoi* pv. *savastanoi*, causal agent of galls or knots on leaves, twigs, and stems of various plant species, were isolated from infected olive (*Olea* sp.), oleander (*Nerium oleander*), jasmine (*Jasminium officinale*), fontanesia (*Fontanesia phillyreoides*) and myrtle (*Myrtus communis*) plants growing in Adana, Antalya, Bursa, Canakkale, Hatay, Izmir, Mersin, Mugla, Samsun and Tekirdag provinces of Turkey. Among the strains initially tested, 33 of them from different hosts were selected for further analyses. The strains were Gram-, oxidase-, pectolytic activity-, levan-, and arginine dihydrolase negative, showed fluorescence on King's B and PVF-1 media and induced hypersensitive reaction on tobacco leaves. Three products 464 bp, 684 bp and 1064 bp in size were amplified by PCR. Fatty acid methyl ester profiles confirmed the identity of tested strains as *P. savastanoi* pv. *savastanoi*. Based on cluster analysis of fatty acids, the strains were divided into two different groups. No correlation was found, however, between host plant and geographical origin of the strains under study. Olive, oleander, jasmine, fontanesia and myrtle plants were used to determine pathogenicity of the strains, which were generally most virulent when inoculated to the original host. This is the first detailed study showing that *P. savastanoi* pv. *savastanoi* can cause knot disease on the new oleaceous host *Fontanesia phillyreoides.*

Key words: *Pseudomoanas savastanoi*, olive, oleander, jasmine, fontanesia, myrtle, FAME, PCR.

INTRODUCTION

Pseudomonas savastanoi pv. *savastanoi* is a pathogenic bacterium inducing galls or knots on stem, twigs, and leaves of several host plants belonging to the family Oleaceae and the related family Apocynaceae (Janse, 1981). According to current classification, three different pathovars of *P. savastanoi* denoted *savastanoi, nerii* and *fraxini*, elicit hyperplastic growth formations (galls or knots) on young stems and branches of olive (*Olea* spp.), oleander (*Nerium oleander*), and ash (*Fraxinus excelsior*), respectively (Young *et al*., 1996). Earlier studies have reported the occurrence of this disease on olive (Smith, 1908; Young *et al*., 1978), oleander (Wilson, 1965); jasmine [*Jasminium officinale* (Janse, 1981)], ash [*Fraxinus excelsior* (Janse, 1981)], privet [*Ligustrum japonicum* (Bottalico and Ercolani, 1971)], *Forsythia* and *Phyllyire*a (Iacobellis *et al*., 1998; Alvarez *et al*., 1998).

The presence of bacterial knot in Turkey was observed on olives growing in the Aegean (Azeri, 1993; Tatli and Benlioglu, 2004) and western Mediterranean (Basim and Ersoy, 2000) regions of the country and on oleander in Sanliurfa (south-eastern Anatolia) (Kavak and Ustun, 2009). Hyperplasia of affected tissues that evolves into clearly visible knots is induced by indolacetic acid (IAA) and cytokinins produced by the pathogen (Smidt and Kosuge, 1978; Surico *et al*., 1985; Iacobellis *et al*., 1994). Bacterial strains from different host plants can be distinguished on the account of differential characteristics related to pathogenicity, biochemical features, genome structure and production of phytohormones (Surico *et al*., 1985; Surico and Iacobellis, 1992). Janse (1991) divided *P. savastanoi* pv. *savastanoi* strains into three homogenous groups, based on the fatty acids profiles and host plant interactions.

The objective of the present study was to characterize *P. savastanoi* pv. *savastanoi* strains from olive, oleander, jasmine, fontanesia (a hitherto unknown host of the bacterium) and myrtle plants growing in the Turkish provinces of Adana, Antalya, Bursa, Canakkale, Hatay, Izmir, Mersin, Mugla, Samsun and Tekirdag on the basis of morphological, physiological and biochemical tests, hypersensitive reaction (HR) on tobacco, pathogenicity tests, fatty acid methyl ester (FAME) profiles, and genetic fingerprinting.

MATERIALS AND METHODS

Isolation. Knotted samples form olive, oleander, jas-

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Table 1. Primers used and DNA amplification conditions.

Primer	Sequence 5'-3'	Amplification conditions	Reference
		35 cycles	
IAALF	GGCACCAGCGGCAACATCAA	94°C 30 sec	Penyalver *et al.*
IAALR	CGCCCTCGCAACTGCCATAC	62°C 30 sec	(2000)
		72°C 30 sec	
PSS1	TGGGGTGCTACTTGTACCCGA	30 cycles	Basim and Ersoy,
PSS2	CCGTGTACTACGTTCAGCGAG	95°C 30 sec	(2000);
PSS3	CAGGACTTCAGAACCCACGT	62°C 30 sec	Ersoy (2002)
PSS4	CGGTCGATGATGTAGAGCAT	72°C 45 sec	

mine, fontanesia and myrtle plants were collected from the above mentioned areas and bacteria were isolated on King's medium B [KB (King *et al.*, 1954)] and PVF1 medium (Alvarez *et al.*, 1998). Greenish-grey young knots from infected plants were macerated in 1 ml of sterile distilled water. Suspensions were streaked on the media, incubated at 25°C for two days and maintained in a refrigerator for 24 h to inhibit the development of saprophyte bacteria. Single, weak fluorescent, creamy colonies were selected and purified on KB.

Pathogenicity tests. Pathogenicity tests were carried out as described by Surico *et al.* (1984). A total of 33 strains were inoculated into 1-year-old shoots of olive, jasmine, fontanesia and myrtle and in 3-month-old shoots of oleander. Bacterial suspensions (10^8 CFU ml^{-1}) of selected bacterial strains were injected in three replicates into the bark of each test plant using a sterile needle. Knot development was observed up to 60-70 days post inoculation, when bacterial strains were re-isolated from symptomatic plants. Sterile distilled water and reference *P. savastanoi* pv. *savastanoi* strain CFPB 1672 from olive (provided by Dr. Cindy Morris, INRA France) were used as negative and positive controls, respectively.

Biochemical and physiological tests. Morphological properties on PVF1 (Surico and Lavermicocca, 1989) and KB (King *et al.*, 1954) media and biochemical and nutritional tests were used to identify the bacteria., i.e. Gram reaction, levan production on 5% saccharose nutrient agar (SNA), oxidase and pectolytic activity, arginine dehydrolase reaction, and HR test on tobacco leaves (Schaad *et al.*, 2001). All tests were repeated twice with three replicates. The reference strain CFPB 1672 was used as positive control.

PCR assays. *Genomic DNA isolation.* Total genomic DNA from bacterial strains was isolated according to De Boer and Ward (1995). Bacterial strains were grown in 9 ml liquid nutrient broth for 24 h at 25°C. An aliquot of 1 ml of bacterial suspension was centrifuged at 14,000 *g* for 20 min, then 100 µl 1% SDS + TAE buffer were added to the pellets and vortexed. Tubes were kept for 3 h in a water bath at 50°C whereafter 50 µl of 7.5 M ammonium acetate were added, mixed and centrifuged at 14,000 *g* for 15 min. The upper aqueous phase was transferred into a new Eppendorf tube, and an equal volume of freezer chilled isopropanol (2-propanol) was added and mixed gently until the DNA precipitated. The tubes were kept in a freezer for 45 min, then centrifuged at 10,000 *g* for 10 min. Finally, pellets were washed in 100 µl of 70% cold ethanol and centrifuged for 10 min at 10,000 *g*. Pellets were saved and resuspended in 50 µl double-distilled sterile water. Efficiency of extraction and DNA quality were assessed by electrophoresis in a 1% agarose gel at 70 V for 1 h and staining with ethidium bromide.

PCR assays. These assays were performed using *P. savastanoi* pv. *savastanoi*-specific primers IAALF-IAALR designed by Penyalver *et al.* (2000) and Pss1-Pss2 and Pss3-Pss4 designed by Basim and Ersoy (2000) and Ersoy (2002). PCR runs were carried out in a 25 µl final volume containing 2.0 µl of DNA template, 2.0 µl of a 10 pmol solution of each primer, 12.5 µl Master Mix (Promega M7502, USA), and 6.5 µl nuclease-free water. All amplifications were performed in a Techne Thermocycler (TC-412, UK) repeating runs three times. Amplicons electrophoresed in 1.5% agarose gel were stained with ethidium bromide (10 mg $µl^{-1}$) and photographed using an Uvipor gel documentation system (125.0.0) (Uvitec). DNA ladders of 1 kb and 100 bp (Fermentas, Germany) were used as markers.

Genetic relationship. The primers for BOX-, REP- and ERIC-PCR (Louws *et al.*, 1994; Norman *et al.*, 2003) and conditions for each technique are shown in Table 2. All amplifications were carried out in 25 µl final volume, containing 3 µl of DNA template, 1.5 µl 20 pmol of each primer, 12.5 µl Master Mix (Promega M7502, USA) and 6.5 H_2O for ERIC- and REP-PCR.

Table 2. Primers used for rep-PCR and DNA amplification conditions.

Method	Primer	Sequence 5'-3'	Amplification conditions
BOX -PCR	BOXA-1R	CTACGGCAAGGCGACGCTGACG	30 cycles 94°C 1min 53°C 1 min 65°C 8min
ERIC-PCR	ERIC 1R ERIC 2	ATGTAAGCTCCTGGGGATTCAAC AAGTAAGTGACTGGGGTGAGCG	30 cycles 94°C 1 min 52°C 1 min 65°C 8 min
REP-PCR	REPIR-1 REP2-1	IIIICGICGICATCIGGC ICGICTTATCIGGCCTAC	30 cycles 94°C 1 min 44°C 1 min 65°C 8 min

Conditions for BOX-PCR were 8.0 H_2O, containing 3 µl of DNA template, 1.5 µl 20 pmol of each primer and 12.5 µl Master Mix (Promega M7502, USA). Amplicons were recovered, stained and photographed as above. All data were subjected to statistical analysis using the Statistical Package for Social Sciences (SPSS, USA) to analyze genetic relationships.

Whole cell fatty acid analysis. For fatty acid analysis, 19 bacterial strains were transferred and grown onto trypticase soy broth agar (TSBA) medium. Analysis of gas chromatography results was made using the TSBA60 database and fatty acid analysis was done using the Sherlock Microbial Identification System (MIDI, USA) with Sherlock 3.6 software (Sasser, 1990).

Cluster analysis. Phenotypic relationships among selected bacterial strains were determined by statistical analysis of the relative quantities of identified fatty acids in each single strain. Cluster analysis techniques were used to produce unweighted pair matching based on fatty acid composition (Guven *et al.*, 2004). Standard deviations were calculated with the SPSS version 5.5a.

RESULTS AND DISCUSSION

Thirty three bacterial strains were isolated from typical galls of olive, oleander, myrtle (Fig. 1A), fontanesia (Fig. 1B) and jasmine (Fig. 1C) plants (Table 3). All showed fluorescence on PVF1 and King B medium, were Gram-negative, did not produce levan on SNA, were negative for pectolytic activity, arginin dihydrolase and oxidase, therefore were allocated in the LOPAT 1b

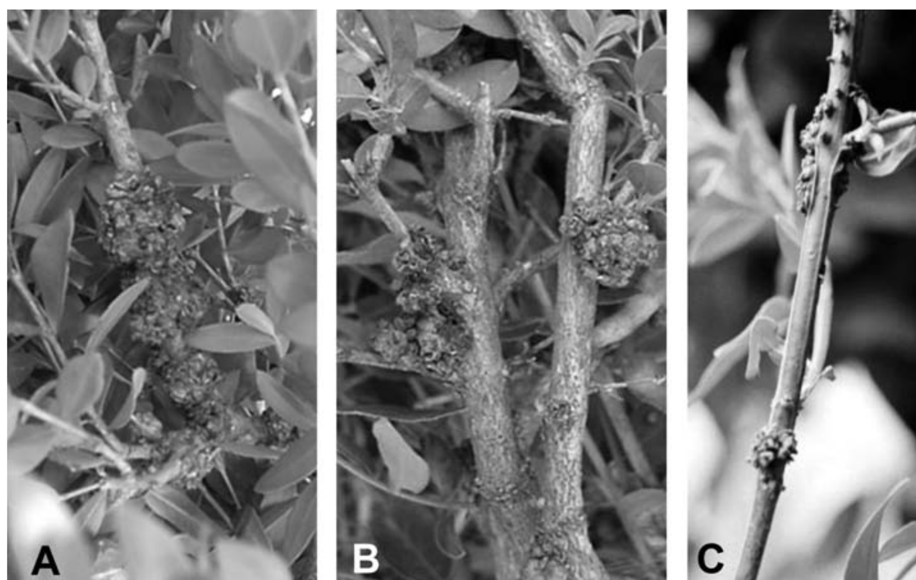

Fig. 1. Galls on myrtle (A), fontanesia (B) and jasmine (C), naturally infected by *Pseudomonas savastanoi* pv. *savastanoi.*

Table 3. List of *Pseudomonas savastanoi* pv. *savastanoi* strains used in this study.

Number	Designation No.	Host Plant	Location
1	Olive 1	*Olea* sp.	Adana
2	Olive 2	*Olea* sp.	Adana
3	Olive 3	*Olea* sp.	Hatay
4	Olive 4	*Olea* sp.	Hatay
5	Olive 5	*Olea* sp.	Tekirdag
6	Olive 6	*Olea* sp.	Tekirdag
7	Olive 7	*Olea* sp.	Canakkale
8	Olive 8	*Olea* sp.	Canakkale
9	Oleander 1	*Nerium oleander*	Mugla
10	Oleander 2	*Nerium oleander*	Antalya
11	Oleander 3	*Nerium oleander*	Adana
12	Oleander 4	*Nerium oleander*	Adana
13	Oleander 5	*Nerium oleander*	Mersin
14	Oleander 6	*Nerium oleander*	Mersin
15	Oleander 7	*Nerium oleander*	Samsun
16	Oleander 8	*Nerium oleander*	Bursa
17	Oleander 9	*Nerium oleander*	Izmir
18	Oleander 10	*Nerium oleander*	Samsun
19	Oleander11	*Nerium oleander*	Bursa
20	Oleander 12	*Nerium oleander*	Bursa
21	Jasmine 1	*Jasminium officinale*	Adana
22	Jasmine 2	*Jasminium officinale*	Adana
23	Jasmine 3	*Jasminium officinale*	Mersin
24	Jasmine 4	*Jasminium officinale*	Mersin
25	Jasmine 5	*Jasminium officinale*	Izmir
26	Jasmine6	*Jasminium officinale*	Izmir
27	Myrtle 1	*Myrtus communis*	Adana
28	Myrtle 2	*Myrtus communis*	Adana
29	Myrtle 3	*Myrtus communis*	Adana
30	Myrtle 4	*Myrtus communis*	Hatay
31	Fontanesia 1	*Fontanesia phillyreoides*	Adana
32	Fontanesia 2	*Fontanesia phillyreoides*	Adana
33	Fontanesia 3	*Fontanesia phillyreoides*	Adana

group of fluorescent pseudomonads (Lelliott and Stead, 1987).

All strains from fontanesia (Fig. 2) and myrtle produced knots on all host species tested whereas olive, oleander and jasmine strains showed host preference and did not induce galls on some species. For example, olive strains were not pathogenic to oleander, whereas oleander strains caused disease to olive, as previously reported (Janse, 1981; Surico *et al*., 1984). Likewise, strains from oleander did not form knots on jasmine and vice versa. In accordance with previous reports (Janse, 1981, 1982, 1991; Young *et al*., 1991), strains were generally more virulent on the original than on the alternative hosts (Fig. 3A, B and C). The original bacterial isolates were

Table 4. Pathogenicity tests results of all *Pseudomonas savastanoi* pv. *savastanoi s*trains isolated and tested in this study on different hosts.

	Olive (8) strains	Nerium (12) strains	Jasmine (6) strains	Myrtle (4) strains	Fontanesia (3) strains	CFBP 1672	Negative control
O. europaea	+++	+	+	+	++	+	-
N. oleander	-	+++	-	+	+	-	-
J. revolutum	++	-	+++	+	+	+	-
M. communis	+	+	+	+++	+	+	-
F. phillyreoides	+	+	+	+	+++	+	-

Results were evaluated 9 weeks after inoculation and recorded as + according to the average size of knots measured for all strains tested on different hosts. -: no gall production or only slight callus formation; +: 4-10 mm knots; ++: 11-20 mm knots; +++: 21-35 mm knots.

Fig. 2. Galls induced by of *Pseudomonas savastanoi* pv. *savastanoi* on fontanesia (*Fontanesia phillyreoides*) following artificial inoculation.

successfully recovered from artificially infected plants. The identity of the 19 selected *P. savastanoi* strains was confirmed by amplification of species-specific sequences that yielded amplicons of the expected size, 464 bp (Fig. 4) and 684 bp (Fig. 5) with primer sets IAALF-IAALR and Pss1-Pss2 and Pss3-Pss4, respectively, in accordance with previous reports (Penyalver *et al.*, 2000; Basim and Ersoy, 2001; Ersoy, 2002).

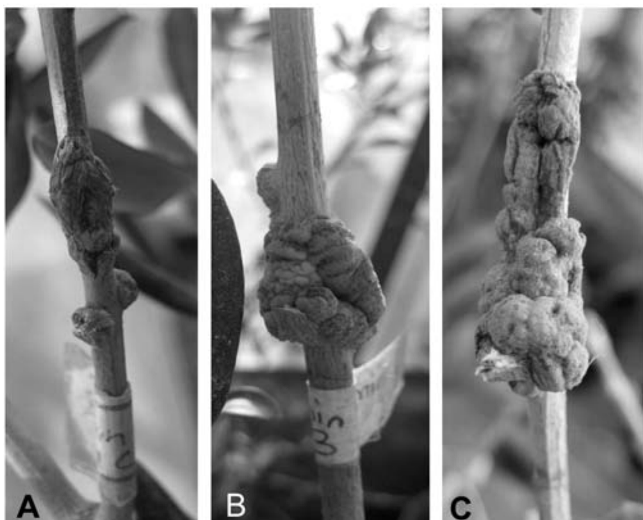

Fig. 3. Results of pathogenicity tests with *savastanoi* pv. *savastanoi*. A, 7 mm knot produced by strain myrtle 2; B, 16 mm knot produced by strain jasmine 3; C, 24 mm knot produced by strain olive 2.

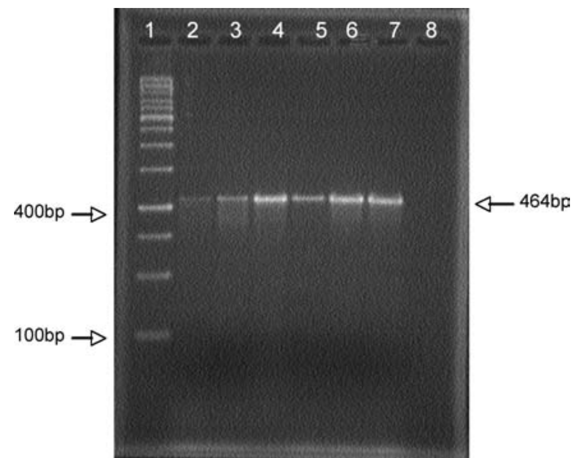

Fig. 4. PCR amplification with IAALF-IAALR primer set of *Pseudomonas savastanoi* pv. *savastanoi* strains from different host plants. Lane 1, 100 bp molecular marker; lane 2, CFPB 1672; lane 3, olive 1; lane 4, oleander 1; lane 5, myrtle 1; lane 6, jasmine 1; lane 7, fontanesia 1; lane 8, negative control.

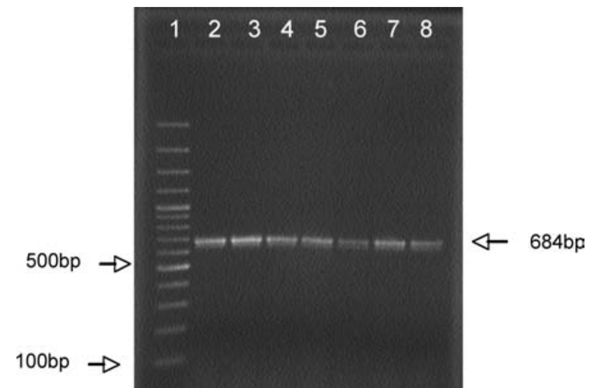

Fig. 5. PCR amplification with PSS1-PSS2 primer set of *Pseudomonas savastanoi* pv. *savastanoi* strains from different host plants. Lane 1 100-bp molecular marker; lane 2, CFPB 1672; lanes 3-4, olive 1 and 2; lane 5, oleander 1; lane 6, myrtle 1; lane 7, jasmine 1; lane 8, fontanesia 1.

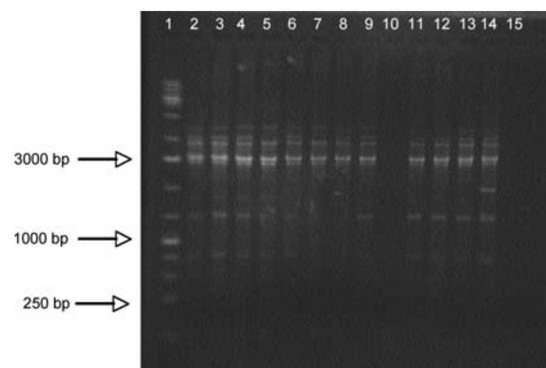

Fig. 6. PCR amplification with BOX A1R of *Pseudomonas savastanoi* pv. *savastanoi* strains from different host plants. Lane 1, 1 kb molecular marker; lane 2, CFPB 1672; lanes 3-5, olive 1, 2, and 3; lanes 6-9, oleander 1, 2, 3 and 4; lanes 10-12, myrtle 1, 2 and 3; lane 13, jasmine 1; lane 14, Fontanesia 1; lane 15, negative control.

Table 5. The results of fatty acid analysis of *Pseudomonas savastanoi* pv. *savastanoi* strains.

Fatty Acids	No. of Strains	Range	Mean	Standard Derivation
10:0 3OH	23	2.94-5.81	3.57	0.63
12:0	25	2.88-6.11	4.30	0.92
12:0 2OH	23	0.66-3.38	2.85	0.59
12:0 3OH	23	3.70-5.49	3.99	0.42
14:0 ISO	1	0.10	0.10	-
14:0	11	0.14-0.66	1.11	1.66
unknown 14.502	2	0.42-1.29	0.86	0.62
Sum in feature2	2	7.31-9.03	8.22	1.22
15:0	1	0.61	0.61	-
15:0 ISO	1	0.41	0.41	-
15:0 ANTEISO	2	1.08-4.44	2.76	2.38
16:0 ISO	1	1.04	1.04	0.63
Sum in feature2	2	7.31-9.03	8.22	0.92
15:0	1	0.61	0.61	0.59
15:0 ISO	1	0.41	0.41	0.42
15:0 ANTEISO	2	1.08-4.44	2.76	-
16:0 ISO	1	1.04	1.04	2.41
Sum in feature3	25	26.51-38.90	35.36	-
16:1 w5c	1	0.15	0.15	2.02
16:0	25	23.72-31.82	26.66	0.27
17:0 ANTEISO	2	0.31-0.69	0.50	1.25
17: CYCLO	15	059-5.48	1.89	0.12
16:0 3OH	2	0.35-0.52	0.44	-
17:0	1	0.43	0.43	-
Sum in feature5	1	0.34	0.34	2.48
18:1 w7c	25	12.71-25.00	19.54	2.28
18:1 w9c	3	0.20	2.79	0.49
18:0	21	0.38	1.09	0.22
11 methyl 18:1 w7c	12	0.35	0.71	0.31
Sum in feature7	8	0.33	0.71	2.28

Summed Feature 2	12:0ALDE
Summed Feature 3	16:1 w7c /15 ISO 2OH
Summed Feature 5	18:2w6,9c/18:0ANTE
Summed Feature7	Unknown 18.846/19:1 w6c

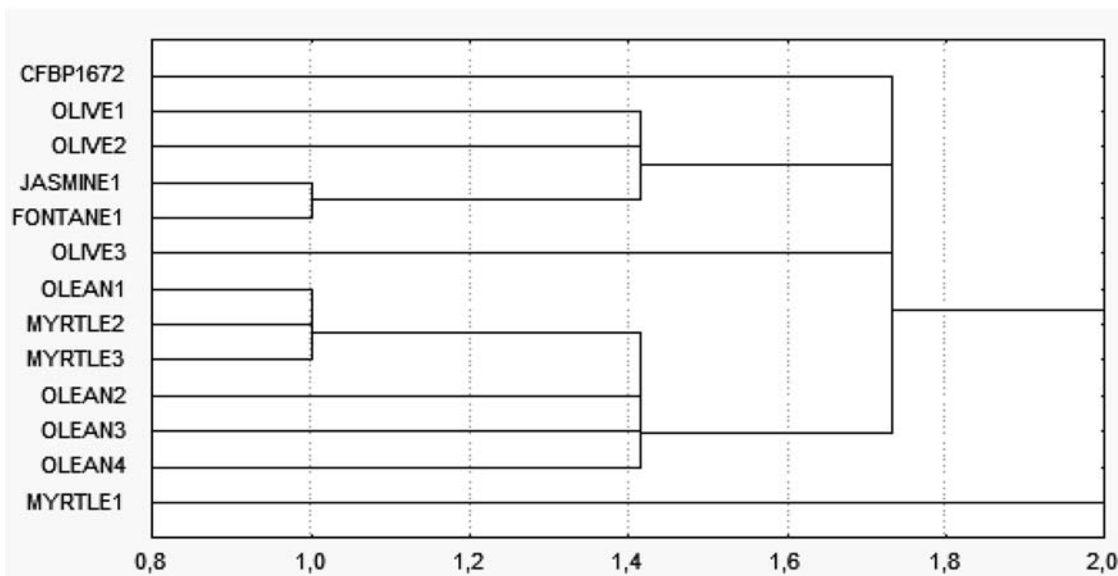

Fig. 7. Cluster analysis of BOX-PCR of *Pseudomonas savastanoi* pv. *savastanoi* strains from different host plants.

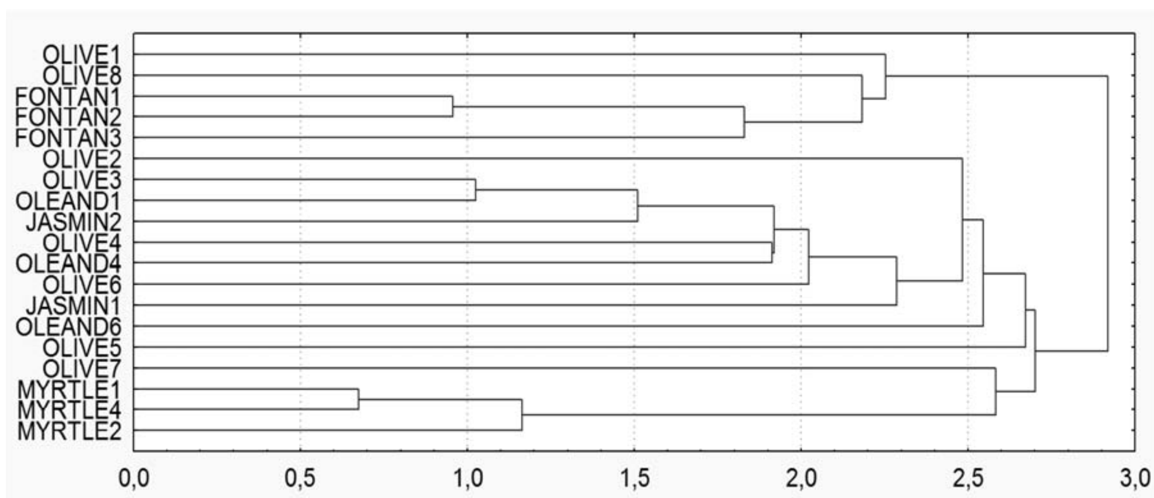

Fig. 8. Cluster analysis of fatty acids of *Pseudomonas savastanoi* pv. *savastanoi* strains from different host plants.

The BOXA1R primer was the most suitable for showing polymorphism in *P. savastanoi* pv. *savastanoi*. By contrast, repeated testing of ERIC- and REP-PCR products did not not disclose any polymorphism among the bacterial strains under study. As shown in Fig. 6, with BOX-PCR all strains generated bands of 2,900, 3,000 bp and 3,400 bp. Some, however, exhibited additional bands with sizes of 1,500 bp and 3,500 bp (10 strains), or 750 and 3,800 bp (9 strains) or 2,000 bp (only one strain). Interestingly, BOX-PCR profiles of the strains from the same host were unique and identical (Fig. 7), except for one from olive (Olive 3) and myrtle (Myrtle 1), which were out of two main clusters.

As to genetic relationship, the similarity level among isolates determined with BOX-PCR was 98% for isolates from olive, oleander, jasmine and fontanesia, whereas myrtle strains showed no less than 99% similarity (Fig. 7).

Bacterial strains were also identified by FAME analysis, showing similarity indices with the entry of *P. savastanoi* in the standard MIS library ranging from 34 to 90%. The patterns obtained had more than 98% similarity (Fig. 8). Similarly to BOX-PCR, two clusters of strains were identified by FAME, one of which included isolates form olive, jasmine, fontanesia and oleander and the other only those from myrtle. There was no apparent relationship between strains isolated from different hosts and their geographical origin.

Even though three different *P. savastanoi* pathovars (‘*savastanoi*’, ‘*nerii*’ and ‘*fraxini*’) pathogenic to olive oleander and ash are known (Young *et al*., 1996), our results show that there are no differences in terms of FAME analysis and BOX-PCR between bacterial isolates recovered from olive, jasmine, fontanesia, oleander and myrtle.

The detailed comparison of *P. savastanoi* pv. *savastanoi* strains from different hosts in Turkey has clearly shown that knot diseases observed in these hosts are all caused by closely related *P. savastanoi* pv. *savastanoi* strains, as determined by phenotypical and genotypical fingerprints and pathogenicity tests. Thus, the present study substantiates the results of an earlier report (Mirik *et al*., 2006) and provides evidence that *P. savastanoi* pv. *savastanoi* is the causal agent knot disease of fontanesia.

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