Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Characterization of bacterial knot disease caused by *Pseudomonas savastanoi* pv. *savastanoi* on pomegranate (*Punica granatum* L.) trees: a new host of the pathogen

I.A. Bozkurt[1], S. Soylu[1], M. Mirik[2], C. Ulubas Serce[3] and Ö. Baysal[4]

1 Department of Plant Protection, Agriculture Faculty, Mustafa Kemal University, Antakya, Hatay, Turkey
2 Department of Plant Protection, Agriculture Faculty, Namık Kemal University, Tekirdag, Turkey
3 Department of Plant Production & Technologies, Faculty of Agricultural Science and Technologies, Nigde University, Nigde, Turkey
4 Department of Molecular Biology & Genetic, Faculty of Life Sciences, Muğla Sıtkı Koçman University, Muğla, Turkey

Significance and Impact of the Study: Pomegranate trees were affected by the disease with outgrowths (galls or knot) disease. Currently, there is no published study on disease agent(s) causing the galls or knots on pomegranate trees in worldwide. Bacterial colonies were isolated from young knots. The causal agent of the knot *Pseudomonas savastanoi* pv.*savastanoi* (*Psv*) was identified based on symptoms, biochemical, molecular methods, pathogenicity tests and sequence analysis. To the best of our knowledge, this is the first report of *Psv* on pomegranate as a natural host, which extends the growing list of plant species affected by this bacterium in the world and Turkey.

Keywords
bacterial knot, PCR, pomegranate, *Pseudomonas savastanoi* pv. *savastanoi*, Turkey.

Correspondence
Soner Soylu, Mustafa Kemal University, Department of Plant Protection, Agriculture Faculty, 31034 Antakya, Hatay, Turkey.
E-mail: soylu@mku.edu.tr

2014/0357: received 20 February 2014, revised 15 July 2014 and accepted 15 July 2014

doi:10.1111/lam.12309

Abstract

This study aimed to isolate and identify the causal organism causing hyperplastic outgrowths (knots) on stems and branches of pomegranate trees in the Eastern Mediterranean region of Turkey. Bacterial colonies were isolated from young knots on plates containing selective nutrient media. Biochemical tests, fatty acid analysis and PCR were performed to identify possible causal disease agent. Representative isolates were identified as *Pseudomonas*.pv.*savastanoi* (*Psv*) using biochemical tests, fatty acid profiling and PCR. Following inoculation of pomegranate plants (cv. hicaz) with bacterial suspensions, 25 of 54 bacterial isolates caused typical knots at the site of inoculation. PCR analysis, using specific primer for *Psv*, generated a single amplicon from all isolates. The similarity of the sequence of Turkish pomegranate isolate was 99% similar to the corresponding gene sequences of *Psv* in the databases. Based on symptoms, biochemical, molecular, pathogenicity tests and sequence analyses, the disease agent of knots observed on the pomegranate trees is *Psv*. To the best of our knowledge, this research has revealed pomegranate as a natural host of *Psv*, which extends the list of host plant species affected by the pathogen in the world and Turkey.

Introduction

The pomegranate (*Punica granatum* L.) is one of the important fruit trees grown in several countries and has nutritional values because of the presence of several bioactive compounds in its different parts. Fruits contain active phytochemicals such as sterols, terpenoids, fatty acids, anthocyanins and procyanidins (Seeram *et al.* 2006). The native range of pomegranate production area spans from Iran to the Central Asia and then spreads east and west through hot, arid regions of India, Asia Minor and the Mediterranean coast (Holland *et al.* 2009). Pomegranate is grown as a common backyard crop in Turkey. In recent years, there has been an increased interest in the commercial production of the fruit in several regions of Turkey, where 270 000 Da of land are under pomegranate cultivation with the production of 315 150 tonnes (Anonymous 2012). Global production has substantially

 © 2014 The Society for Applied Microbiology

increased in the past decade, and pomegranate is being consumed not only as a fresh fruit, but also as juice or concentrated sour syrup. Hatay province is one of the main production areas of pomegranates in Turkey, where 10 531 Da of land is under pomegranate cultivation with the production of 15 543 tonnes (Anonymous 2012).

Plant diseases caused by a range of fungal agents are known to be the major problems in the cultivation of pomegranate in several countries including Turkey (Pala *et al.* 2009; Somasekhara *et al.* 2011; Gat *et al.* 2012). There is, however, limited information about the bacterial disease(s) specific to pomegranate trees growing worldwide. Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is a major constraint for pomegranate production in important pomegranate-growing countries such as India, Pakistan and South Africa (Akhtar and Bhatti 1992; Petersen *et al.* 2010; Mondal *et al.* 2012).

Upon complains of pomegranate growers in Hatay province, brief surveys were conducted to determine plant disease problems encountered by the growers in the pomegranate orchards. The presence of hyperplastic outgrowths (as seen round galls or knots) on stems, branches and twigs of pomegranate plants was recorded in several orchards in the districts of Hatay province. The symptoms caused on pomegranate trees closely similar to those induced by the *Pseudomonas savastanoi* pv. *savastanoi* (hereafter termed as *Psv*) on olive (*Olea europea*) and other related host species (Gardan *et al.* 1992; Ramos *et al.* 2012). As pomegranate orchards have already been established nearby the olive orchards in the region, it was assumed that the bacterial disease agent of olive knot could also be the causal agent of the symptoms observed on pomegranate stems and branches. Therefore, plant materials with symptoms were collected to investigate the reason for the disease of pomegranate orchards in the region.

The aim of this study was to isolate and identify the disease agent causing hyperplastic outgrowths on stems, branches and twigs of pomegranate trees in the surveyed regions.

Results and discussion

The presence of hyperplastic outgrowths was observed on pomegranate stems, twigs and branches during disease surveys carried out in 2011 and 2012 in the main pomegranate-growing areas. The highest incidence was recorded up to 65% in several orchards in the two districts of Hatay province (Yayladagı and Arsuz). The disease symptoms were typically characterized with several outgrowths appearing either singly or close together. These outgrowths were often observed as a single mass that could extend up to 10 cm along the stems. Symptoms ranged from small swellings to smooth spherical green knots increasing in size (up to 3·0 cm in diameter) as they matured. Old infections were typically characterized as darker and more furrowed appearances. Knots were mostly inconspicuous and in some cases girdled the branch (Fig. 1).

Bacterial colonies were isolated from several pomegranate trees representing two different locations on nutrient media NSA, PVF-1 and KB. Fifty-four isolates from naturally infected knots were isolated and subjected to biochemical and pathogenicity tests. These isolates were mostly nonfluorescent or produced very weak fluorescent colonies on KB or PVF-1, as the fluorescence was lost after the first subculturing. On PVF-1 medium, most of the isolates grew slowly, small, greyish-white, slightly raised colonies with entire margins as reported previously

Figure 1 Typical galls (arrows) induced by *Pseudomonas savastanoi* pv. *savastanoi* on naturally infected pomegranate tree (cv. hicaz). Note that appearance of galls ranged from smooth spherical knots (a) to darker and more furrowed (b–d) appearances as they matured.

(Surico and Lavermicocca 1989). The reactions of these bacterial isolates to Levan production, oxidase, potato soft rot and arginine dihydrolase tests were all negative. Among the 54 isolates, 25 bacterial isolates from naturally infected pomegranate trees caused necrosis (the hypersensitive reaction) 24 h after inoculation on tobacco leaves. The obtained results were similar to those of the reference isolates (Mirik and Aysan 2011). Cultures from galls having a necrotic appearance were predominantly occupied by a pale yellowish secondary bacterial isolate. According to Marchi *et al.* 2006; *Pantoea agglomerans* was found associated with the pathogen *Psv* in 70% of the olive knots examined. Similar observation was also reported for olive knot disease survey which was conducted in Turkey (Mirik and Aysan 2011).

Pathogenicity tests were conducted using 54 pomegranate isolates from the pure cultures obtained from the knots, those isolated from olive knots on olive trees in the same locations and nonpathogen *Pseudomonas syringae* pv. *phaseolicola* (as a negative control). Following inoculation of pomegranate plants (cv. hicaz) with bacterial suspensions, 25 of 54 bacterial isolates caused typical symptoms of bacterial knots caused by *Psv* within 3 weeks after inoculation, like those observed with the olive isolates of *Psv*. Formation of the knots started at the inoculation sites as swelling and then enlarged in size forming fleshy tissues (Fig. 2a). Inoculation of olive (cv. Gemlik) and oleander plants with pomegranate isolate (HNP4) was also included in the pathogenicity tests. The pomegranate isolate has produced typical knot formation only on olive plants

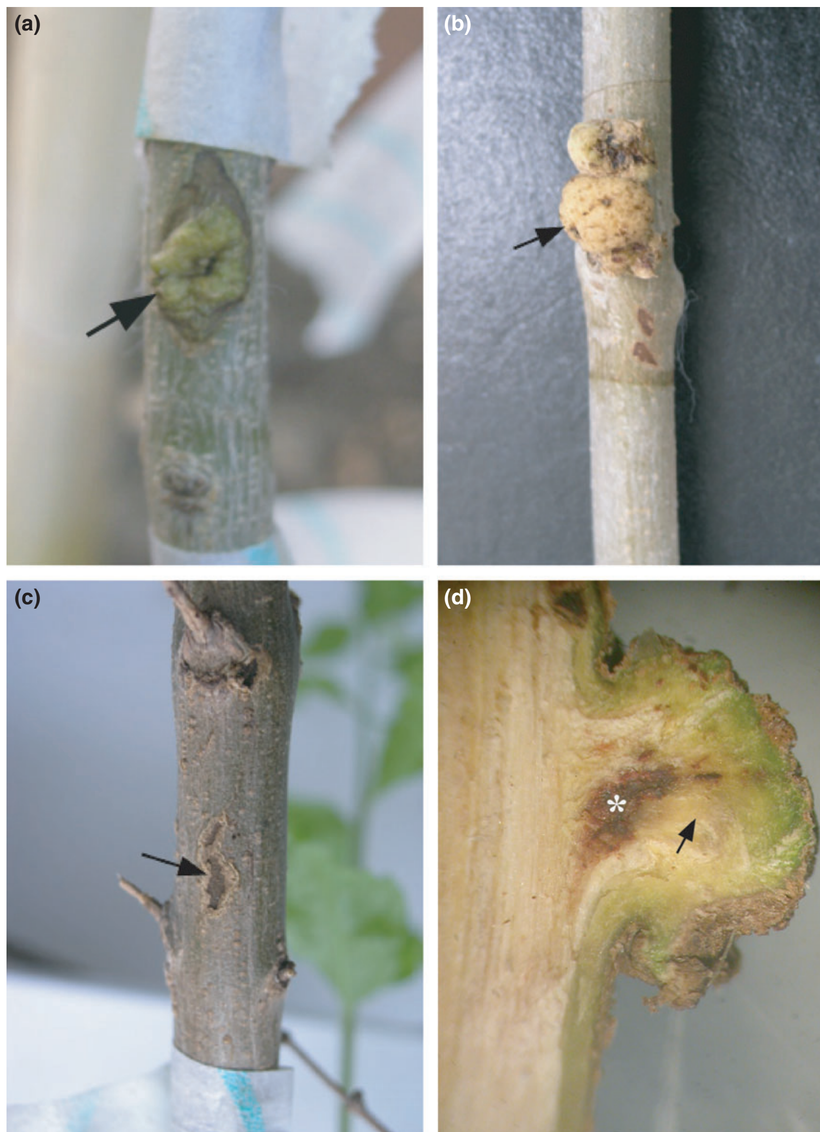

Figure 2 Typical galls formation (arrow) at the site of inoculation on (a) pomegranate (cv. hicaz) and (b) olive (cv. Gemlik) plants artificially inoculated by pomegranate isolate HNP4. (c) Shows negative control without gal formation at the site of inoculation (arrow) on pomegranate (cv. hicaz) plant artificially inoculated by *Pseudomonas syringae* pv. *phaseolicola*. (d) Shows necrotic area (*) that is developed in gall and surrounded by glassy water soaked tissue (arrow), 2 months after inoculation.

4 weeks after inoculation (Fig. 2b). No disease symptoms were recorded on pomegranate trees inoculated with sterile water, bean pathogen *Ps. syringae* pv. *phaseolicola* (Fig. 2c). Two months after inoculation, all bacterial isolates were re-isolated from knots produced at the inoculation sites on pomegranate stems (Fig. 2d) and compared with the original cultures used for inoculation. Bacteria isolated from symptomatic pomegranate plants had retained the characteristics of the isolate initially used for inoculation. These re-isolated bacterial isolates were further used for identification using PCR and fatty acid methyl ester (FAME) analysis. FAMEs have proved and been recognized as useful biochemical markers for bacterial classification and characterization (Weyent *et al.* 1996). Following pathogenicity test, 25 representative re-isolates from different knot samples from pomegranate were identified as *Psv* based on fatty acid profiles (similarity index ranged between 0·57 and 0·76; MIS-RTSBA, ver. 6.0, MIDI Inc., Newark, DE). Fatty acid composition slightly varied for *Psv* isolates from pomegranate. According to results of FAME analysis, twenty-five different fatty acids were detected in pomegranate isolates of *Psv* (Table 1). The fatty acid profiles of pomegranate and olive isolate of *Psv* revealed that more than 76% of the total peak area of all cellular fatty acids occurring in all bacterial isolates was accounted for by three major components: 16:1 w7c, 16:0 and 18:1 w7c. The hydroxy-substituted acids 10:0 3OH, 12:0, 12:0 2OH, 12:0 3OH and 17:0 cyclo contributed on average a further 20·4%. Fatty acid compositions of *Psv* isolates from pomegranate were compared with the reference isolate of *Psv* from olive plants (Table 1). Pomegranate isolates of *Psv* were different from olive isolate. Six minor fatty acid components, 11:0 iso 3OH, 11:0 3OH, 13:0, 16:0 iso, 17:0 anteiso and 18:0 iso, were found in only pomegranate isolates. Four minor fatty acid components, 12:1 3OH, 16:0 3OH, 18:1:w7c 11-methyl, 19:0 cyclo w10c, were found in olive isolates of *Psv* but not in pomegranate isolate of *Psv*.

Specific PCR assays were performed using the primer pairs *Psv*F/*Psv*R using genomic DNA of *Psv* isolates. Genomic DNA of pomegranate and olive isolates was used as template, and result obtained is shown in Fig. 3. Sixteen pomegranate isolates were arbitrarily selected along with reference olive isolate. A single 388-bp product was obtained from all *Psv* isolates from pomegranates and olives, showing that enterobacterial repetitive intergenic consensus (ERIC) sequences (Tegli *et al.* 2010) were present in isolates from both hosts (Fig. 3). For comparison, virulent isolates of *Ps. syringae* pv. *phaseolicola* (bean pathogen), taxonomically related to *Psv*, were also included in the study. Genomic DNA from *Ps. syringae* pv *phaseolicola* species did not produce expected band upon amplification (Fig. 3). For comparison, pathovar-specific PCR assays were also conducted using primer pairs *Psn*F/*Psn*R and *Psf*F/*Psf*R which are specific the *Pseudomonas savastanoi* pv. *nerii* and *Pseudomonas savastanoi* pv. *fraxini* (Tegli *et al.* 2010) to discriminate *Psv* from these taxonomically related bacterial disease agents. Genomic DNA from isolates of *Psv* from pomegranate and olive did not produce expected bands upon amplification with primer pairs *Psn*F/*Psn*R and *Psf*F/*Psf*R (Fig. 4).

Sequence of the PCR amplicon from bacterial isolate HNP4, as a representative pomegranate isolate, was submitted to GenBank (accession number KJ453118). A BLAST analysis on this sequence showed that this isolate belongs to the *Psv*. The similarity of the sequences of Turkish pomegranate isolate was 99% identical to the corresponding gene sequences of *Psv* ERIC-clone for

Table 1 Fatty acid methyl ester (FAME) profiles (%) of bacterial isolates obtained from knots caused by *Pseudomonas savastanoi* pv. *savastanoi* from pomegranate and olive plants

Retention time	Fatty acid	Host plant	
		Pomegranate	Olive
1·27	10:0	0·11	0·15
1·58	10:0 3OH	2·91	3·60
1·71	12:0	4·90	4·63
1·74	11:0 iso 3OH	0·10	–
1·83	11:0 3OH	0·06	–
1·98	13:0	0·07	–
2·04	12:0 2OH	3·16	3·25
2·08	12:1 3OH	–	0·12
2·12	12:0 3OH	4·59	5·34
2·28	14:0	0·26	0·24
2·79	16:0 iso	0·14	–
2·86	16:1 w7c	31·12	32·40
2·89	16:1 w5c	0·12	0·12
2·91	16:0	25·36	25·40
3·11	17:0 iso	0·47	0·30
3·14	17:0 anteiso	0·07	–
3·17	17:1 w8c	0·28	0·27
3·20	17:0 cyclo	4·84	2·87
3·23	17:0	0·62	0·42
3·41	16:0 3OH	–	0·57
3·43	18:0 iso	0·13	–
3·47	18:2 w6	0·12	0·36
3·50	18:1 w7c	19·55	18·84
3·53	18:1 w5c	0·09	0·11
3·55	18:0	0·78	1·14
3·58	18:1:w7c 11-methyl	–	0·33
3·82	19:0 cyclo w10c	–	0·13
3·84	19:0 cyclo w8c	0·72	0·20
3·86	19:0	0·06	0·27
	Similarity indices (RTSBA 6·0 Sherlock)	0·762	0·699

– indicates absence of the fatty acid in bacterial isolate.

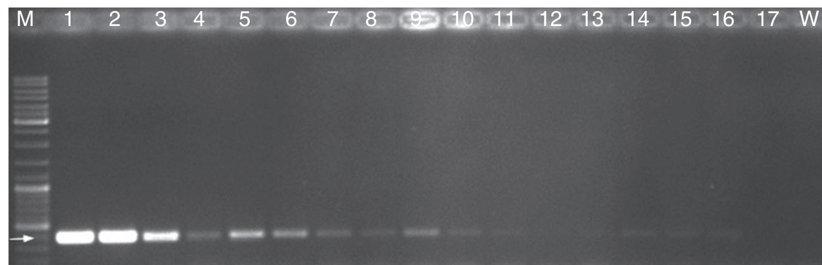

Figure 3 Amplification of DNA fragment from the genomic DNA of different isolates of *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) with primer set *Psv*F/*Psv*R. Lane M: 10 Kb molecular weight marker; lanes 1–2 PCR product from reference olive isolates of *Psv*; lanes 3–16: PCR product from different pomegranate isolates of *Psv*; lanes 17: absence of PCR product from the nontarget isolate of *Pseudomonas syringae* pv. *phaseolicola*; lane W, DNA-free negative control (water). Arrow shows amplification of a 388-bp DNA fragment.

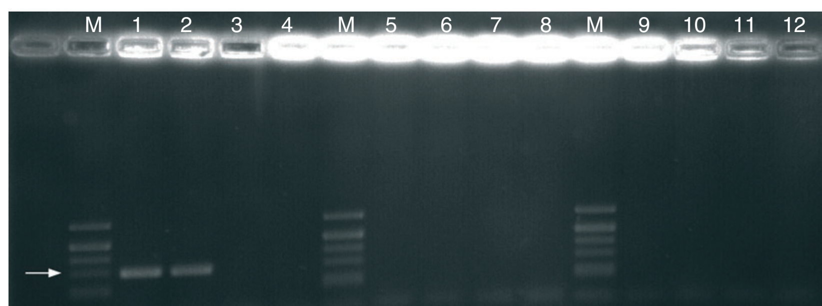

Figure 4 Amplification of the genomic DNA of pomegranate and olive isolates of *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) with different primer set. Lane M: 1 Kb molecular weight marker; lanes 1–4 Amplification of genomic DNA of selected isolates with primer set *Psv*F/*Psv*R; lanes 5–8 Amplification of genomic DNA of selected isolates with primer set *Psn*F/*Psn*R; lanes 9–12 Amplification of genomic DNA of selected isolates with primer set *Psf*F/*Psf*R. Note that single amplicon was only produced with the primer set *Psv*F/*Psv*R. Lanes 1, 5, 9 PCR product from reference olive isolates of *Psv;* Lane 2, 6, 10 PCR product from pomegranate isolates of *Psv;* Lanes 3, 7, 11 shows absence of PCR product from the nontarget isolate of *Pseudomonas syringae* pv. *phaseolicola.* lanes 4, 8, 12, show DNA-free negative control (water). Arrow shows amplification of a 388-bp DNA fragment.

pathovar-specific PCR detection, clone 1 present in the databases (accession number FM253089).

Pathovars of *P. savastanoi* are bacterial pathogens to occur on common ash, olive, oleander, and privet (Gardan *et al.* 1992; Ramos *et al.* 2012). Bacterial isolates from olive, oleander and ash have been identified as *Psv*, *Ps. savastanoi* pv. *nerii* and *Ps. savastanoi* pv. *fraxini*, respectively (Young *et al.* 1996). In previous studies, olive isolates cause typical knot symptoms on olive and Oleaceae, oleander isolates cause disease symptoms on oleander and plants of Oleaceae, but ash isolates cause disease symptoms only on ash plants (Janse 1982; Surico *et al.* 1985; Iacobellis *et al.* 1998). *Psv* was recently reported to occur on *Osmanthus fragrans* and *Loropetalum chinense* (Cinelli *et al.* 2013; Conner *et al.* 2013). Bacterial knot disease agent *Psv* is the most important bacterial diseases of olive trees in the Mediterranean countries including Turkey (Tegli *et al.* 2010; Mirik and Aysan 2011). Disease agent has been also detected in Australia, USA, Asia, North Africa and the Middle East (Hall *et al.* 2004; Balestra *et al.* 2008; Alabdalla *et al.* 2009; Krid *et al.* 2009).

Although pomegranate is natural host of several fungal diseases, bacterial leaf blight caused by *X. axonopodis* pv. *punicae* is the only bacterial disease of pomegranate in the world (Akhtar and Bhatti 1992; Petersen *et al.* 2010; Poovarasan *et al.* 2013). Disease agent has been reported as the reason for reduction of the domestic and export pomegranate production by 80% in India (Ravikumar *et al.* 2011; Poovarasan *et al.* 2013). Currently, there is no published study for the galls or knots caused by disease agent(s) or *Psv* on pomegranate trees worldwide. Based on symptoms on the pomegranate plants, in combination with the results of biochemical, molecular, pathogenicity tests and sequence analyse, it can be concluded that the causal agent of the knot symptoms on the pomegranate trees growing in the region is *Psv*.

To the best of our knowledge, this is the first report of pomegranate as natural host of *Psv*, which extends the growing list of cultivated plant species affected by this

phytopathogenic bacterium in the world and Turkey. The origin of the disease infection has not been reported. The infection could be disseminated from the nearby olive trees by pruning tools as olive trees are widely cultivated in the region. Additional surveys are being continued in other provinces of Turkey to determine the prevalence and incidence of the disease.

Material and methods

Isolation of bacterial isolates

The samples were randomly obtained by collecting young soft knots with smoother surface of stems and branches of pomegranate trees in major growing area during period of 2011 and 2012. Total of 54 pomegranate isolates were isolated from fresh pomegranate knots taken from 7 orchards located at three different districts. Each knot was processed separately. Young knots were first washed under running tap water to remove adhering soil and dust particles, surface disinfected by dipping into 2·5% (v/v) sodium hypochlorite for 2–3 min. and rinsed with sterile distilled water (SDW), placed to dry on sterile filter paper and finally disinfected with cotton moistened with ethanol. Small fragments (5–10 mm) from each knot were aseptically cut with a sterile scalpel and placed in a sterile eppendorf tube containing 1 ml sterile saline (0·85% NaCl) and allowed to sit for 10 min. Tenfold serial dilutions were spread on plates containing King's medium B (KB) (King *et al.* 1954) and PVF-1 agar (Surico and Lavermicocca 1989) and incubated at 26°C for 3 days. Single fluorescent or nonfluorescent representative colonies of the predominant morphological types of bacterial isolates were re-streaked onto new KB and PVF-1 plates and incubated at 26°C for 3 days. The pure single colonies obtained were grown on KB slants and stored at 4°C in 20% glycerol at –80°C until further identification. Two virulent *Psv* isolates, obtained from olive trees growing in the same location (HZP2) and reference isolate (Havran2b) used in a previous study (Mirik and Aysan 2011), were also included for comparison.

Identification of bacterial isolates

Biochemical analysis

Selected representative bacterial isolates from pomegranate and the reference isolate (Havran2b) of *Psv* from olive were subjected to biochemical tests for characterization and identification. The biochemical tests such as levan production, oxidase, potato soft rot, arginine dihydrolase and tobacco hypersensitivity (LOPAT) were conducted according to Lelliott and Stead (1987).

Fatty acid composition

Isolates of *Psv* were streaked onto trypticase soy broth agar (TSBA) and grown for 24 h at 28°C. Cellular fatty acids were extracted and derivatized to their fatty acid methyl esters (FAMEs) as described by Janse (1991). FAMEs were separated by the Microbial Identification System (MIDI Microbial ID, Inc., Newark, NJ) utilizing an Agilent Technologies 6890N GC with a G2614A autosampler and a 7683 injector. FAME peaks were analysed using MIDI, Sherlock software ver. RTSBA 6.0.

Pathogenicity tests

Pathogenicity test was performed on 1-year-old pomegranate plants cv. Hicaz using the most virulent pomegranate isolate (HNP4) according to method described by Mirik and Aysan (2011). Bacterial suspensions (10^8 CFU ml^{-1}) from the cultures obtained from typical knots on naturally infected pomegranate plants were inoculated into wounds made by a sterile scalpel on the bark of 1-year-old pomegranate stems. Wounds were wrapped with parafilm for 3 days. The inoculated plants were enclosed in a plastic bag to maintain near 100% humidity for 24 h. The bags were then removed, and the plants were placed in a growth chamber with a relative humidity ranged from 80 to 85% and a temperature of 26°C and watered twice a week. For comparison, two virulent *Psv* isolates from olive (HZP2 and Havran2b) were used as positive control. Plants were also inoculated with sterile distilled water and bean pathogen *Ps. syringae* pv. *phaseolicola* (as a negative control). The pathogenicity of the pomegranate isolate (HNP4) was also performed on 1-year-old olive (cv. Gemlik) and oleandar plants as described above.

Specific PCR

Bacterial DNA was extracted from liquid cultures (10^{10} CFU ml^{-1}) using the GeneJET™ Genomic DNA Purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. Identification of bacterial isolates was performed using the specific polymerase chain reaction (PCR) described by Tegli *et al.* (2010). In the PCR assay, primer pair, *Psv*F (5-GGCGATGTTCTCAGCGGATTTG-3) and *Psv*R (5-GA TCAAGTGTCCAAGGAAGTGAAGG-3), which was designed from enterobacterial repetitive intergenic consensus (ERIC) sequences, was used and produced a fragment of 388 bp. For discrimination of *Psv* from taxonomically closely related disease agents *Ps. savastanoi* pv. *nerii* (oleander knot) and *Ps. savastanoi* pv. *fraxini* (ash knot), specific primer pairs *PsnF*/*PsnR* (*Psn*F ACCCCTCA TTGTAACGGATG/*Psn*R TCCCCGGAATTCAACACTTA) and *PsfF*/*PsfR* (*Psf*F CGCCTGCTGTACTCCTCGG/*Psf*R

TCGACCTGTCTAAGGCC) were also used (Tegli *et al.* 2010). PCR amplifications were carried out in a 50-μl reaction mixture.

PCR amplifications were performed in a thermal cycler (Thermo Arktik 5020; Thermo Fisher Scientific, Vantaa, Finland), under the published conditions (Tegli *et al.* 2010). Negative (DNA-free sterile water and *Ps. syringae* pv. *phaseolicola*) controls were included in all PCR amplifications to test for contaminants in the reagents used. For each bacterial isolate, amplification reactions were performed at least twice, in three separate experiments.

The PCR products were sequenced in both forward and reverse directions using an automatic DNA sequencer (ABI 3100, Applied Biosystems). The sequences were compared with those available in the MegaBlast program (http://www.ncbi.nlm.nih.gov/).

Conflict of Interest

We declare that there is no conflict of interest exists for this study.

References

Akhtar, M.A. and Bhatti, M.H.R. (1992) Occurrence of bacterial leaf spot of pomegranate in Pakistan. *Pak J Agric Res* **13**, 95–97.

Alabdalla, N., Valentini, F., Moretti, C., Essa, S., Buonaurio, R. and Abu-Ghorra, M. (2009) First report of *Pseudomonas savastanoi* pv. *savastanoi* causing olive knot in Syria. *Plant Pathol* **58**, 1170.

Anonymous. (2012) TUIK, Bitkisel üretim istatistikleri veri tabani. http://tuikapp.tuik.gov.tr/bitkiselapp/bitkisel.zul

Balestra, G.M., Lamichhane, J.R., Kshetri, M.B., Mazzaglia, A. and Varvaro, L. (2008) First report of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi* in Nepal. *Plant Pathol* **58**, 393.

Cinelli, T., Rizzo, D., Marchi, G. and Surico, G. (2013) First report of knot disease caused by *Pseudomonas savastanoi* on sweet olive in central Italy. *Plant Dis* **97**, 419.

Conner, K.N., Olive, J., Zhang, L., Jacobi, J. and Putnam, M.L. (2013) First report of bacterial gall on *Loropetalum chinense* caused by *Pseudomonas savastanoi* in the United States. *Plant Dis* **97**, 835.

Gardan, L., Bollet, C., Abu-Ghorrah, M.A., Grimont, F. and Grimont, P.A.D. (1992) DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. *Int J Syst Bacteriol* **42**, 606–612.

Gat, T., Liarzi, O., Skovorodnikova, Y. and Ezra, D. (2012) Characterization of *Alternaria alternata* causing black spot disease of pomegranate in Israel using a molecular marker. *Plant Dis* **96**, 1513–1518.

Hall, B.H., Coter, E.J., Whattman, M., Noble, D., Luck, J. and Cartwright, D. (2004) First report of olive knot disease on olives (*Olea europea*) in Australia. *Australas Plant Pathol* **33**, 433–436.

Holland, D., Hatib, K. and Bar-Ya'akov, I. (2009) Pomegranate: botany, horticulture, breeding. *Hortic Rev* **35**, 127–192.

Iacobellis, N.S., Caponero, A. and Evidente, A. (1998) Characterization of *Pseudomonas syringae* ssp. *savastanoi* strains isolated from ash. *Plant Pathol* **47**, 73–83.

Janse, J.D. (1982) *Pseudomonas syringae* subsp. *savastanoi* (ex Smith) subsp. nov., nom. rev., the bacterium causing excrescences on Oleaceae and *Nerium oleander* L. *Int J Syst Bacteriol* **32**, 166–169.

Janse, J.D. (1991) Pathovar discrimination within *Pseudomonas syringae* subsp. *savastanoi* using whole-cell fatty acids and pathogenicity as criteria. *Syst Appl Microbiol* **13**, 79–84.

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**, 301–307.

Krid, S., Rhoum, A., Quesada, J.M., Penyalver, R. and Gargouri, A. (2009) Delineation of *Pseudomonas savastanoi* pv. *savastanoi* strains isolated in Tunisia by random-amplified polymorphic DNA analysis. *J Appl Microbiol* **106**, 886–894.

Lelliott, R.A. and Stead, D.E. (1987) Methods for the diagnosis of bacterial diseases of plants. In *Methods in Plant Pathology*, vol. 2, ed. Preece, T.F. pp. 176–199. Oxford: Blackwell Scientific Publications.

Marchi, G., Sisto, A., Cimmino, A., Andolfi, A., Cipriani, M.G., Evidente, A. and Surico, G. (2006) Interaction between *Pseudomonas savastanoi* pv. *savastanoi* and *Pantoea agglomerans* in olive knots. *Plant Pathol* **55**, 614–624.

Mirik, M. and Aysan, Y. (2011) Phenotypic and genotypic characterization of *Pseudomonas savastanoi* pv. *savastanoi* isolates and disease prevalence of olive knot disease in Marmara Region of Turkey. *J Agric Sci* **17**, 279–290.

Mondal, K.K., Rajendran, T.P., Phaneendra, C., Mani, C., Sharma, J., Pooja, R.S., Verma, G., Kumar, R. *et al.* (2012) The reliable and rapid polymerase chain reaction (PCR) diagnosis for *Xanthomonas axonopodis* pv. *punicae* in pomegranate. *Afr J Microbiol Res* **6**, 5950–5956.

Pala, H., Tatli, A., Yilmaz, C. and Özgüven, A.I. (2009) Important disease of pomegranate fruit and control possibilities in Turkey. *Acta Hortic* **818**, 285–290.

Petersen, Y., Mansvelt, E.L., Venter, E. and Langenhoven, W.E. (2010) Detection of *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight on pomegranate in South Africa. *Australas Plant Pathol* **39**, 544–546.

Poovarasan, S., Mohandas, S., Paneerselvam, P., Saritha, B. and Ajay, K.M. (2013) Mycorrhizae colonizing actinomycetes promote plant growth and control bacterial blight disease

of pomegranate (*Punica granatum* L. cv Bhagwa). *Crop Prot* **53**, 175–181.

Ramos, C., Matas, I.M., Bardaji, L., Aragón, I.M. and Murillo, J. (2012) *Pseudomonas savastanoi* pv. *savastanoi*: some like it knot. *Mol Plant Pathol* **13**, 998–1009.

Ravikumar, M.R., Wali, S.Y., Benagi, V.I., Patil, H.B. and Patil, S.S. (2011) Management of bacterial blight of pomegranate through chemicals/antibiotics. *Acta Hortic* **890**, 481–482.

Seeram, N.P., Schulman, R.N. and Heber, H. (2006) *Pomegranate: Ancient Roots to Modern Medicine*. Boca Raton, FL: CRC Press, Taylor & Francis Group.

Somasekhara, Y.M., Kale, R.D. and Hosmath, J.A. (2011) Effect of culture filtrates of vermicompost against pomegranate (*Punica granatum* L.) wilt pathogen, *Ceratocystis fimbriata* Ell. & Halst. *Res Crop* **12**, 217–221.

Surico, G. and Lavermicocca, P. (1989) A semiselective medium for the isolation of *Pseudomonas syringae* pv. *savastanoi*. *Phytopathology* **79**, 185–190.

Surico, G., Iacobellis, N.S. and Sisto, A. (1985) Studies on the role of indole-3-acetic acid and cytokinins in the formation of knots on olive and oleander plants by *Pseudomonas syringae* pv. *savastanoi*. *Physiol Plant Pathol* **26**, 309–320.

Tegli, S., Cerboneschi, M., Marsili, I., Libelli, I.M. and Santilli, E. (2010) Development of a versatile tool for the simultaneous differential detection of *Pseudomonas savastanoi* pathovars by End Point and Real-Time PCR. *BMC Microbiol* **10**, 156–169.

Weyent, R.S., Moss, C.W., Weaver, R.E., Hollis, D.G., Jordan, J.G., Cook, E.C. and Daneshvar, M.I. (1996) *Identification of Unusual Pathogenic Gram-negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd edn. Baltimore, MD: Williams & Wilkins.

Young, J.M., Saddler, G.S., Takikawa, Y., De Boer, S.H., Vauterin, L., Gardan, L., Gvozdyak, R.I. and Stead, D.E. (1996) Names of plant pathogenic bacteria 1864–1995. *Rev Plant Pathol* **75**, 721–763.