Eur J Plant Pathol (2016) 146:837–846
DOI 10.1007/s10658-016-0962-2

Isolation and characterization of *Pseudomonas syringae* pv. *syringae* which induce leaf spot on walnut

Ali Reza Keshtkar · Gholam Khodakaramian · Kiomars Rouhrazi

Accepted: 11 May 2016 / Published online: 16 May 2016

Abstract In 2012, a leaf spot disease on walnut seedlings was observed in Hamedan province of Iran. The spots were necrotic with yellow halos. Symptomatic samples were collected and suspected bacterial agent was isolated on nutrient agar medium. Phenotypic characteristics such as production of fluorescent pigment on KB medium, LOPAT test and utilization of various carbon sources, revealed that the strains were *Pseudomonas syringae* pv. *syringae*. All tested strains were pathogenic on peach, plum and walnut seedling. To investigate the genetic diversity among the strains, rep-PCR using BOX and ERIC primers was performed. Results showed high similarity among the strains from the same region, while variation was found among those from different areas and they were divided into two groups based on geographic regions. The phylogenetic analysis based on 16S rRNA, *rpoD* and *gyrB* sequences showed that representative isolates were closely related to *P. syringae* pv. *syringae*. To the best of our knowledge, this is the first report of the presence of *P. syringae* pv. *syringae* as a causal agent of walnut seedlings leaf spot in Iran and worldwide.

Keywords Walnut · *Pseudomonas syringae* pv. *syringae* · 16S rRNA · BOX-PCR · ERIC-PCR · Iran

A. R. Keshtkar · G. Khodakaramian (✉) · K. Rouhrazi
Department of Plant Protection, Faculty of Agriculture, University of Bu-Ali Sina, Hamedan, Iran
e-mail: Khodakaramian@yahoo.com

Introduction

Walnut species (*Juglans* sp.) are important nut and timber producer's intemperate regions of Europe, Asia and North America. The Persian walnut (*Juglans regia*) is the most horticultural developed and widely cultivated and is easily the leading producer of commercial nuts (Hajri et al. 2010). Therefore diseases affecting this plant will cause important economic losses worldwide. Here we report a spot disease characterized by the formation of necrotic zones or halos in leaves of walnut seedlings in Iran that resembles those produced in other plants by *Pseudomonas syringae* pv *syringae*.

The phytopathogenic *Pseudomonads* cause numerous plant diseases with diverse symptoms including cankers, diebacks, blossom, twig, leaf or kernel blights, leaf spots (*Pseudomonas syringae* pathovars), soft or brown rots (*P. viridiflava* and *P. marginalis* pathovars), tumors or galls (*P. savastanoi* pathovars), and mushroom blights (*P. tolaasii* and *P. agarici*) (Schaad et al. 2001).

P. syringae is a Gram-negative bacterium belonging to the genus *Pseudomonas* sensu stricto, included in the γ subclass of the Proteobacteria (Kersters et al. 1996). Currently *P. syringae* divided into more than 50 pathovars and nine genomic species (Bradbury 1986; Gardan et al. 1999). This bacterium has a wide host range as it causes diseases to over 180 plants.

Pseudomonas syringae pv. *syringae* 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. 2002; Vicente and Roberts 2003, 2007; Gilbert et al. 2009, 2010). 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).

In Iran, phytopatogenic *Pss* strains were isolated from stone fruit trees and other plants in different areas which presented different phenotypic and genotypic characteristics among those isolated from various hosts (Bahar et al. 1982; Banapour et al. 1990; Ashorpour et al. 2008; Abbasi et al. 2013).

During the past decade molecular techniques for identification of plant pathogenic bacteria were developed (Louws et al. 1999). Genomic fingerprinting methods based on the polymerase chain reaction (PCR) have been applied for identification and classification of plant associated bacteria at the subspecies level (Louws et al. 1995, 1999). Repetitive extragenic palindromic (REP) sequences or elements were first described in *Escherichia coli* and *Salmonella typhimurium* operons (Higgins et al. 1982). Repetitive sequences present in the genomes of diverse bacterial species have been used to design PCR primers that generate reproducible fingerprints useful to assess bacterial diversity at the strain and pathovar level (Versalovic et al. 1991, 1994; Louws et al. 1994). The researchers concluded that these methods could be used to identify and classify strains into different pathovars within *Pseudomonas syringae* (Weingart and Volksch 1997). Gutiérrez-Barranquero et al. (2008) and Qing et al. (2011) reported that rep-PCR is an interesting tool for the delineation or genotyping of bacterial species because it is more discriminatory than other DNA fingerprinting techniques. Weingart and Volksch (1997); Vicente and Roberts (2007); Gilbert et al. (2009) and Abbasi et al. (2013) by studying the genetic diversity of *Pss* strains of stone fruits, found that rep-PCR is a rapid and simple method to evaluate of genetic diversity of *Pss* strains and this method also can assist in the identification of *Pss* isolates.

The analysis of 16S rRNA gene sequences has become the standard technique for identification of bacteria (Schaad et al. 2001) because it is highly conserved, but present variations among genera and species of prokaryotes which makes this gene very useful for bacterial identification at genus and species level. Nevertheless, other less conserved genes are more useful to differentiate among phylogenetically close bacterial species.

Phylogenetic relationships of *Pseudomonads* resolved by using *gyrB* (DNA gyrase B subunit) and *rpoD* (σ^{70} factor) sequences were eminently different from those resolved by using 16S rRNA sequences. DNA gyrase is the enzyme responsible for introducing negative supercoils into bacterial chromosomes and plays a crucial role in the replication of chromosomes (Watt and Hickson 1994). The σ^{70} factor, on the other hand, is one of the sigma factors that confer promoter-specific transcription initiation on RNA polymerase (Lonetto et al. 1992).

Yamamoto et al. (2000) reported that the phylogenetic analysis using the *gyrB* and *rpoD* sequences may fill the resolution gap between 16S rRNA sequence analysis and DNA ± DNA hybridization studies.

In this study we isolated for the first time the bacterial strains producing necrotic halos in the leaves of walnut seedlings in Iran. They were characterized by using phenotypic and genetic methods and were identified as *Pseudomonas syringae* pv. *syringae* (*Pss*) by 16S rRNA, *gyrB* and *rpoD* gene sequencing.

Materials and methods

Sampling and isolation of the causal pathogen

During 2012, walnut leave samples shown necrotic lesions were collected from different areas in Hamedan province. Plant samples were washed under tap water, disinfected, washed again with sterilized distilled water, cut into small pieces placed in sterilized distilled water for 30 min. A loopful of suspension was streaked on NA medium and put at 25 °C for 3 days. Each colony was streaked onto NA for to isolate pure single colonies. Purified colonies were streaked on Kings' B medium to detection of fluorescent pigmentation (Hildebrand et al. 1988). The isolates were stored in 30 % (*v*/v) glycerol solution at −80 °C for long-term storage.

Pathogenicity test

Selected isolates were grown on nutrient agar for 48 h at 27 °C. Leaves of walnut seedling were first surface sterilized with 70 % ethanol and rinsed in distilled sterilized water; then, strains were inoculated by depositing 1 μl of a bacterial suspension (10^8 CFU/ml) into leaves with sterilized hypodermic needles. Plants were covered with a plastic bag for 24 h to increase relative humidity and were maintained in a greenhouse at 20–25 °C until the symptoms appeared (Schaad et al. 2001). Also, one ml of bacterial suspensions was injected into the green stems of peach and plum seedlings by using a needle (Little et al. 1998). Each plant was inoculated in ten sites with one strain. Sterilized distilled water was used to inoculate plants that were used as negative controls.

Phenotypic features

Phenotypic features of the bacterial strains were characterized based on standard bacteriological methods. These include; Gram staining and sensitivity to 3 % KOH (Suslow et al. 1982), aerobic growth (Hugh and Leifson 1953) and fluorescent pigment production on King's B medium (Hildebrand et al. 1988). Levan formation, oxidase reaction, potato rot (pectolytic activity), argenine dihydrolase and tobacco hypersensitivity (LOPAT) test was analysed as was previously described (Lelliott et al. 1966). Hydrolysis of gelatin, aesculin and starch, catalase and tyrosinase activities and growth on 4 % NaCl was performed as described by Hildebrand et al. (1988) and growth at 37 °C according to Fahy and Hayward (1983). In addition, utilization of carbon sources was tested on the basal medium of Ayers et al. (1919) supplemented with 0.1 % carbohydrates and amino acids.

DNA preparation

Bacteria were grown on nutrient agar at 27 °C for 48 h. Bacterial suspensions were prepared in sterile distilled water (108 CFU/ml) and were lysed by the addition of 1:10 volume of 3 % KOH and heating the suspension at 95 °C for 2 min with subsequent cooling on ice. Lysates were centrifuged at 8000×g for 2 min, and the supernatants were used directly for PCR or stored at −20 °C until used (Rouhrazi and Rahimian 2012).

Fig. 1 Leaf spot symptoms on walnut seedlings caused by *Psudomonas syringae* pv. *syringae* in Iran

PCR assays using rep-PCR

Amplification reaction was carried out using the total DNA extracted from each isolates as template for ERIC and BOX-PCR. Two specific primers were used to correlate to ERIC sequence (Versalovic et al. 1991). ERIC 1R (5′- ATGTAAGCTCCTGGGGATTCA- 3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGC-3′) (Louws et al. 1994). Also repetitive sequence based PCR typing with the BOX-A1R primer (5′-CTA CGGCAAGGCGACGCTGACG- 3′) (Renick et al. 2008) was carried out as follows. ERIC and BOX-PCR were performed in 25 μl reaction mixtures containing 2.5 μlof 10X PCR buffer, 2.5 mM MgCl2, 0.4 mM dNTPs, 0.2 μlM of each primer, 1.25 U Taq DNA polymerase and 3 μl of template DNA. Amplification was carried out as follows: after initial denaturation for 3 min at 94 °C, 35 amplification cycles were completed, each consisting of 1 min at 94 °C, 1 min at 50 °C (55 °C in BOX-PCR) and 2 min at

Table 1 Bacterial isolates used in this study

Strain	Place of isolation
AK1,AK2,AK3,AK4,AK5,AK6,AK7,AK8,AK9,	Tuyserkan
AK10,AK11,AK12,AK13,AK14, AK15,AK16,AK17,AK18,AK19,AK20	Hamadan

72 °C. A final extension of 10 min at 72 °C was applied (Rademaker and de Bruijn 1997). DNA bands obtained by Eric and Box-PCR and subsequently electrophoresed

Table 2 Phenotypic features of strains of *Pseudomonas syringae* pv. *syringae* isolated from walnut seedling leaf spot in Iran

Characteristics	Reaction
Gram reaction	–
Catalase	+
Fluorescent pigment	+
Levan formation	+
Oxidase	–
Potato rot	–
Arginine dihydrolase	–
HR on Tobacco and Geranium	+
Hydrolysis of starch	+
Ice nucleation	+
Growth at 37° C	–
Growth in 4 % NaCl	+
Grows aerobically	+
Gelatin liquefaction	+
Arbutin hydrolysis	+
Aesculin hydrolysis	+
Utilization of:	
Erythritol	+
L-lactate	+
Glutarate	+
Trigonelline	+
D- arabinose	–
DL-glycerate	+
Trehalose	–
Sorbitol	+
Mannitol	+
Cellobiose	–
Sucrose	+
L-rhamnose	–
Insitol	+
Geraniol	–
Benzoate	–
D(−)-tartrate	–
D-arabinose	–
D-aspartate	–
D-quinate	+
Adonitol	–

+, 80 % or more strains positive; − , 80 % or more strains negative

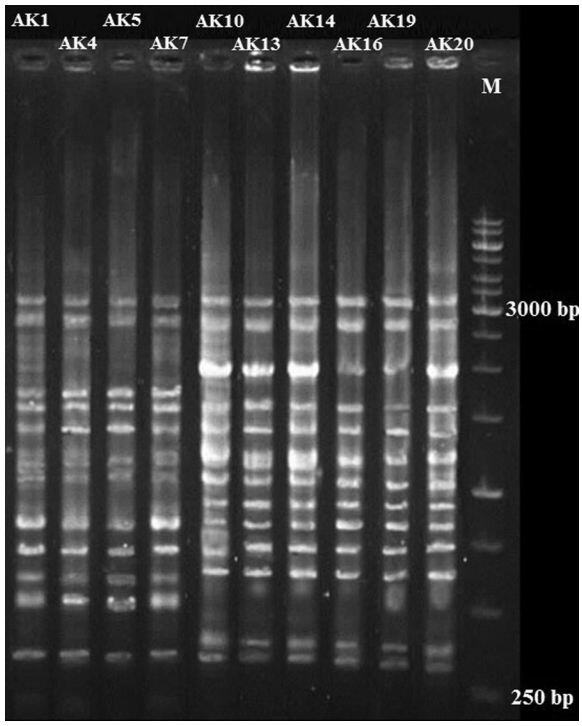

Fig. 2 PCR band pattern using BOXA1 primer for the strains of *Pseudomonas syringae* pv. *syringae* causing walnut seedling leaf spot in Iran. M: 1 kb DNA ladder

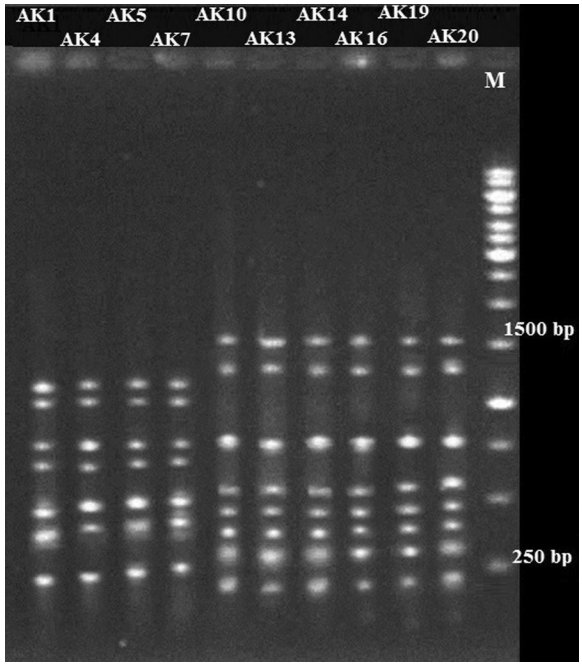

Fig. 3 PCR band pattern using ERIC1/ ERIC2 primers for the strains of *Pseudomonas syringae* pv. *syringae* causing walnut seedling leaf spot in Iran. M: 1 kb DNA ladder

were scored as present (1) or absent (0) and their fingerprints were compared using NTSYS software. Jaccard's coefficient of similarity index was used to calculate similarity distances. Cluster analysis was done using the un-weighted pair-group method with arithmetic average (UPGMA) (Sneath and Sokal 1973).

Sequence analyses of 16S rRNA, *rpoD* and *gyrB* genes

The amplification and sequencing of 16S rRNA, *rpoD* and *gyrB* genes were carried out as 164 described previously (Manceau and Horvais 1997; Sarkar and Guttman 2004). The sequences 165 obtained were compared with those from the GenBank using the BLASTN program (Altschul et al. 1990). The obtained sequences and those of related bacteria obtained from GenBank 167 were aligned using the Clustal W program (Thompson et al. 1997). The distances were 168 calculated according to Kimura's two-parameter model (Kimura 1980). The phylogenetic 169 trees were inferred using the neighbour joining model (Saitou and Nei 1987) and MEGA5.0170 (Tamura et al. 2011) was used for all the phylogenetic analyses.

Results

Isolation and identification of the causal pathogen

From walnut leaf spots (Fig. 1), a total of 46 strains with beige round and smooth margins colonies were isolated on NA medium (Table 1). The isolates were Gram negative, obligate aerobic, and produced fluorescent pigment on King's B medium. They formed levan from sucrose and were oxidase and arginine dihydrolase negative (Table 2). They were potato rot negative and caused hypersensitivity reaction on tobacco and geranium (LOPAT group Ia). Table 2 showed phenotypic properties of the isolates. The isolates induced leaf spots under greenhouse seven days after inoculation and also natural conditions. They were pathogenic on peach and plum seedling and produced progressive necrotic symptom on the inoculated sites of the stems.

The biochemical, physiological and pathogenicity characteristics indicated that the isolates belong to *Pseudomonas syringae* pv. *syringae.*

ERIC-PCR and BOX-PCR analysis

Banding patterns of strains were generated using the BOXA1R and ERIC primers. The bands amplified in the BOX-PCR ranged in length from 250 to 3500 bp (Fig. 2) and those amplified in the ERIC-PCR ranged from 150 to 1500 bp (Fig. 3). Reproducible fingerprint profiles were generated with each technique upon repetition of the procedures. Walnut isolates were differentiated into two clusters at the 90 % similarity level. Group 1 include AK1, AK4, AK5, and AK7 isolated from Tuyserkan and group 2 include AK10, AK13, AK14, AK16, AK19 and AK20 isolated from Hamedan.

16S rRNA, *rpoD* and *gyrB* genes analysis

Based on the analysis of ERIC- and BOX-PCR fingerprints, the isolates (AK1 and AK10) were selected and alignment of their 1400-bp 16S rRNA sequence determined. The sequences have been deposited in the GenBank database under the accession numbers KU321601 and KU321602. Homology searches with the 16S rRNA sequences in the GenBank database showed that the Iranian isolates are 99.5 similar to *Pseudomonas syringae* pv. *syringae*.

The phylogenetic analysis of the 16S rRNA gene sequences of isolated strains within the phylogenetic groups to which belong their closest related pathovars within *Pseudomonas syringae* are shown in Fig. 4.

The nucleotide sequences of 530 bp *ropD* and 550 bp *gyrB* genes were obtained for the representative strains. The analysis of these two genes showed that the strains were phylogenetically close to *P. syringae* pv. *syringae* (Fig. 5).

Discussion

The two most common major species of walnuts are grown for their seeds, the Persian or English walnut and the black walnut. The English walnut (*J. regia*) originated in Persia and the black walnut (*J. nigra*) is native to North America. The purpose of this study was to isolate the bacterium causing leaf spot of walnut (*J. regia*) seedling in Iran and to identify it through the analysis of several biochemical and molecular tests and to confirm its pathogenicity in different plants. In this study, the strains of

causal agent for leaf spot of walnut presented the same phenotypic characteristics (Table 2) and after the application of a set of tests known as LOPAT (Lelliott et al. 1966) for fluorescent *Pseudomonas* group differentiation, the isolates present the typical characteristics of *P. syringae* (formed levan, oxidase and arginine dihydrolase were negative, caused hypersensitivity reaction on tobacco but they were unable to rotten potato slices). Peix et al. (2009) reported phenotypic characteristics such as carbon sources consumption, ability to grow in different culture conditions, antibiotic resistance, producing antibiotics, extracellular enzymes, cell shape and type of flagellum as an easy way to differentiate species from genus *Pseudomonas*. Nevertheless, Lelliott et al. (1966) had showed that among the fifteen tests to determine and differentiate the fluorescent plant pathogenic *Pseudomonas* spp. only a set of tests named as LOPAT could differentiate five distinct pathogenic species groups. Bultreys and Kaluzna (2010) also used LOPAT tests to differentiate of fluorescent plant pathogenic *Pseudomonas* spp. In this study, strains of causal agent for leaf spot of walnut are similar to *Pss* in LOPAT test and consumption of carbon resources (Table 2).

Scortichini et al. (2002) showed that *P. avellanae* and strains similar to *Pss* which are agents of hazelnut decline in northern Greece and central Italy concluded that strains of *Pss* related to hazelnut in LOPAT test are like *P. syringe* but in the consumption of carbon resources such as DL-homoserine and tyrosinase differed from *P. syringe* and have the ability to use these compounds. The authors indicated that the studied strains were a distinct taxon very closely related to *Pss*.

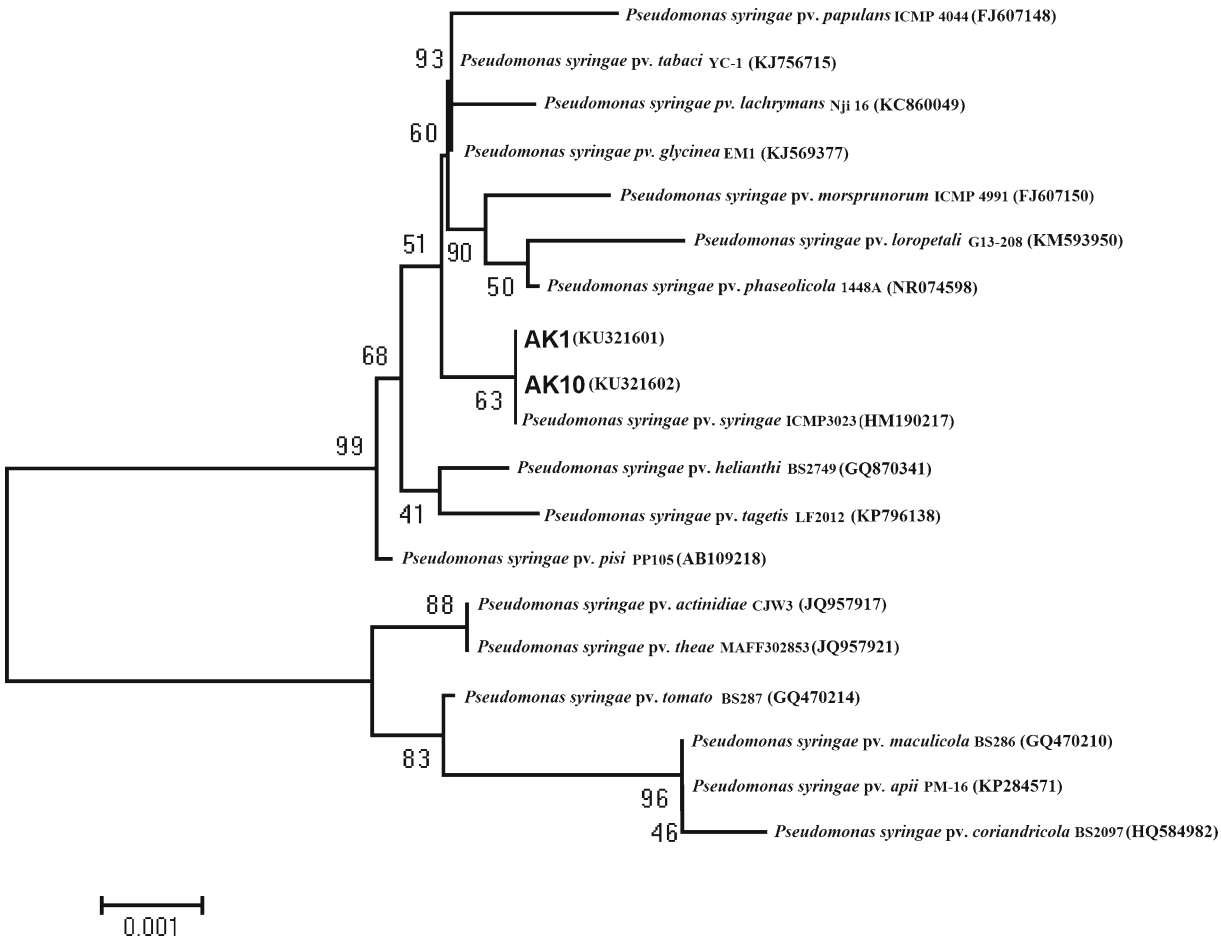

Fig. 4 Neighbor-joining tree based on the 16S rRNA sequences, showing the relationships between the representative walnut isolates from Iran (filled circles) and different pathovars of *Pseudomonas syringae* strains (alignment length 1350 bp). Percentage bootstrap is indicated on the internal branches (replicates)

ERIC and BOX profiles have been used successfully to characterize a large number of bacteria and they differentiate among closely related strains (Versalovic et al. 1991, 1994). These fingerprinting techniques generated characteristic profiles that can be used to differentiate pathogen isolates of *Pseudomonas* from different species and pathovars and even from the same pathovar (Weingart and Volksch 1997; Little et al. 1998; Joana et al. 2007; Marques et al. 2008; Gašić et al. 2012; Kaluzna et al. 2010; Çepni and Gürel 2012; Abbasi et al. 2013). In this work we confirmed that strains from the same pathovar are differentiated by ERIC and BOX fingerprinting technique since different patterns were obtained for strains causing walnut seedling leaf spot isolated in Hamedan province and strains isolated from Tuyserkan, while the strains of the same region showed identical pattern. All these strains showed 99.5 % similarity to *Pss* after the 16S rRNA gene analysis in agreement with the results of the identification based on phenotypic characteristics. Nevertheless, several species of genus *Pseudomonas* have closely related16S rRNA gene sequences and then the analysis of other genes, including *gyrB* and *rpoD*, have been used to study genetic diversity and evolution in *P. syringae* sensu lato and to identify pathovars and strains within this species (Yamamoto et al. 2000; Hwang et al. 2005; Ferrante and Scortichini 2010; Kaluzna et al. 2010; Bull et al. 2011; Martín-Sanz et al. 2012, 2013). The analysis of these two genes in the strains from this study showed that the bacterium causing walnut leaf spot is very close

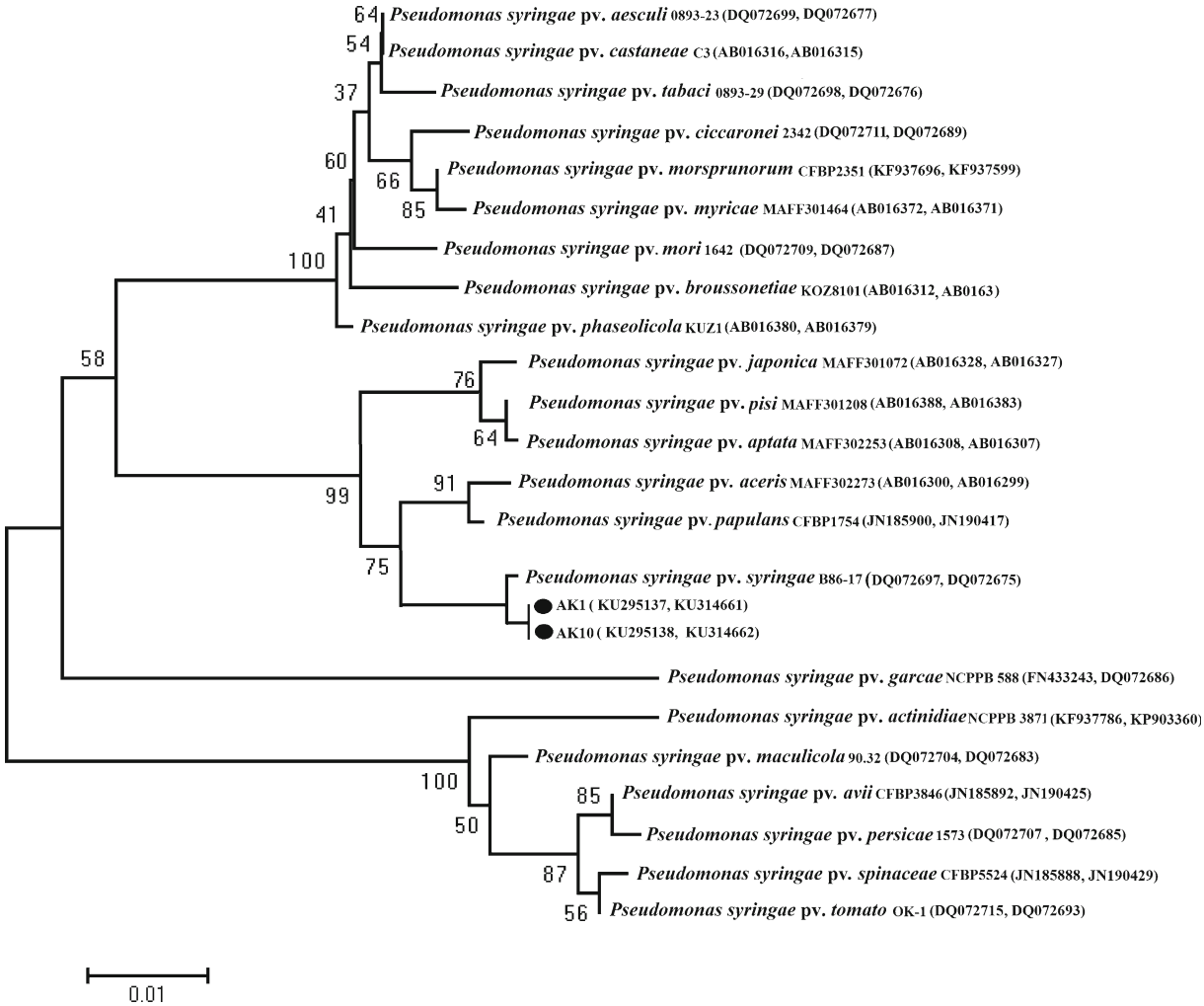

Fig. 5 Neighbour-joining phylogenetic tree based on concatenated *rpoD* and *gyrB* gene sequences of the strains isolated in this study and different pathovars of *Pseudomonas syringae*

to *Pss*. To the best of our knowledge, this is the first report of *Pseudmonas syringae* pv. *syringae* as the causal agent of walnut seedlings leaf spot in Iran and worldwide.

References

Abbasi, V., Rahimian, H., & Tajick-Ghanbari, M. (2013). Genetic variability of Iranian strains of pseudomonas syringae pv. Syringae causing bacterial canker disease of stone fruits. *European Journal of Plant Pathology, 135*, 225–235.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403–410.

Ashorpour, M., Niknejad Kazempour, M., & Ramezanie, M. (2008). Occurrence of *Pseudomonas syringae* pv. *syringae* the causal agent of bacterial canker on olives (*Olea europaea*) in Iran. *Science Asia, 34*, 323–326.

Ayers, S. H., Rupp, P., & Johnson, W. T. (1919). A study of the alkali-forming bacteria in milk. *United States Department of Agriculture Bulletin, 782*, 1–39.

Bahar, M., Mojtahedi, H., & Akhiani, A. (1982). Bacterial canker of apricot in Isfahan. *Iranian Journal of Plant Pathology, 18*, 58–68.

Banapour, A., Zakiee, Z., & Amani, G. (1990). Isolation of *Pseudomonas syringae* from sweet cherry in Tehran Province. *Iranian Journal of Plant Pathology, 26*, 67–72.

Bradbury, J. F. (1986). *Guide to plant pathogenic bacteria*. Kew, United Kingdom: CAB International Mycological Institute.

Bull, C. T., Clarke, C. R., Cai, R., Vinatzer, B. A., Jardini, T. M., & Koike, S. T. (2011). Multilocus sequence typing of *Pseudomonas syringae* sensu lato confirms previously described genomospecies and permits rapid identification of *P. syringae* pv. *coriandricola* and *P. syringae* pv. *apii* causing bacterial leaf spot on parsley. *Phytopathology, 101*, 847–858.

Bultreys, A., & Kaluzna, M. (2010). Bacterial cankers caused by *Pseudomonas syringae* on stone fruit species with special emphasis on the pathovars *syringae* and *morsprunorum* race1 and race2. *Journal of Plant Pathology, 92*, 21–33.

Çepni, E., & Gürel, F. (2012). Variation in extragenic repetitive DNA sequences in *Pseudomonas syringae* and potential use of modified REP primers in the identification of closely related isolates. *Genetics and Molecular Biology, 35*, 650–656.

Fahy, P. C., & Hayward, A. C. (1983). Media and methods for isolation and diagnostic tests. In P. C. Fahy & G. J. Parsley (Eds.), *Plant bacteria diseases, a diagnostic guide* (pp. 337–378). Australia: Academic Press.

Ferrante, P., & Scortichini, M. (2010). Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidiachinensis*) in Central Italy. *Plant Pathology, 59*, 954–962.

Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F., & Grimont, P. A. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *International Journal of Systematic Bacteriology, 49*, 469–478.

Gašić, K., Prokić, A., Ivanović, M., Kuzmanović, N., & Obradović, A. (2012). Differentiation of *Pseudomonas syringae* pathovars originating from stone fruits. *Pesticides and Phytomedicin, 27*, 219–229.

Gilbert, V., Legros, F., Maraite, H., & Bultreys, A. (2009). Genetic analyses of *Pseudomonas syringae* strains from Belgian fruit orchards reveal genetic variability and host preferences within pathovar *syringae*, and help identify both races of pathovar *morsprunorum*. *European Journal of Plant Pathology, 124*, 199–218.

Gilbert, V., Planchon, V., Legros, F., Maraite, H., & Bultreys, A. (2010). Pathogenicity and aggressiveness in populations of *Pseudomonas syringae* from Belgian fruit orchards European journal of plant pathology, 126**,** 23–277.

Gutiérrez-Barranquero, J. A., Arrebola, E., Pérez-García, A., Codina, J. C., Murillo, J., De Vicente, A., & Cazorla, F. M. (2008). Evaluation of phenotypic and genetic techniques to analyze diversity of *Pseudomonas syringae* pv. *syringae* strains isolates frommango trees. In M. B. Fatmi, A. Collmer, N. Iacobellis, J. W. Mansfield, J. Murillo, N. W. Schaad, & M. Ulrich (Eds.), Pseudomonas syringae Pathovars and related pathogens—identification, epidemiology and genomics (pp. 271–281). Netherlands: Springer.

Hajri, A., Meyera, D., Delortb, F., Guillaume'sa, J., Brina, C., & Manceaua, C. (2010). Identification of a genetic lineage within *Xanthomonas arboricola* pv. *juglandis* as the causal agent of vertical oozing canker of Persian (English) walnut in France. Journal of Plant Pathology, 59, 1014–1022.

Higgins, C. F., Ames, G. F. L., Barnes, W. M., Clement, J. M., & Hofnung, M. (1982). A novel intercistronic regulatory element of prokaryotic operons. *Nature, 298*, 760–762.

Hildebrand, D. C., Schroth, M. N., & Sands, D. C. (1988). *Pseudomonas*, *In: N. W. Schaad (Ed.), Laboratory Guide for the identification of plant pathogenic bacteria (pp. 60–80). 2nd edn*. St. Paul, Minnesota, USA: APS Press.

Hugh, R., & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *Journal of Bacteriology, 66*, 24–26.

Hwang, M. S. H., Morgan, R. L., Sarkar, S. F., Wang, P. W., & Guttman, D. S. (2005). Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Applied and Environmental Microbiology, 71*, 5182–5191.

Joana, G., Steven, V., & Roberts, J. (2007). Discrimination of *Pseudomonas syringae* isolates from sweet and wild cherry using rep-PCR. *European Journal of Plant Pathology, 117*, 383–392.

Kaluzna, M., Ferrante, P., Sobiczewiski, P., & Scortichini, M. (2010). Characterization and genetic diversity of *Pseudomonas syringae* from stone fruits and hazelnut using repetitive-PCR and MLST. *Journal of Plant Pathology, 92*, 781–787.

Kersters, K. W., Ludwig, M., Vancanneyt, P., De Vos, M., Gillis, D., & Schleifer, K. H. (1996). Recent changes in the classification of the *Pseudomonads*: an overview. *Systematic and Applied Microbiology, 19*, 465–477.

Kimura, M. A. (1980). Simple method for estimating evolutionary rates of base substitutions through comparative studies of

nucleotide sequences. *Journal of Molecular Evolution, 16*, 111–120.

Lelliott, R. A., Billing, E., & Hayward, A. C. (1966). A determinative scheme for the fluorescent plant pathogenic *Pseudomonads*. *Journal of Applied Microbiology, 29*, 470–489.

Little, E. L., Bostock, R. M., & Kirkpatrick, B. C. (1998). Genetic characterization of *Pseudomonas syringae* pv. *syringae* strains from stone fruits in California. *Applied and Environmental Microbiology, 64*, 3818–3823.

Lonetto, M., Gribskov, M., & Gross, C. A. (1992). The sigma 70 family: sequence conservation and evolutionary relationships. *Journal of Bacteriology, 174*, 3843–3849.

Louws, F. J., Fulbright, D. W., Stephens, C. T., & De Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology, 60*, 2286–2295.

Louws, F. J., Fulbright, D. W., Taylor, S. E., & De Bruijn, F. J. (1995). Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology, 85*, 528–536.

Louws, F. J., Rademaker, J. L., & De Bruijn, F. J. (1999). The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection and diagnosis. *Annual Review of Phytopathology, 37*, 81–125.

Manceau, C., & Horvais, A. (1997). Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato*. *Applied and Environmental Microbiology, 63*, 498–505.

Marques, A. S. A., Marchaison, A., Gardan, L., & Samson, R. (2008). BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae - P. viridiflava* group. *Genetics and Molecular Biology, 31*, 106–115.

Martín-Sanz, A., Pérez de la Vega, M., & Caminero, C. (2012). Resistance to *Pseudomonas syringae* in a collection of pea germplasm under field and controlled conditions. *Plant Pathology, 61*, 375–387.

Martín-Sanz, A., Pérez de la Vega, M., Murillo, J., & Caminero, C. (2013). Strains of *Pseudomonas syringae* pv. *syringae* from pea are phylogenetically and pathogenically diverse. *Phytopathology, 103*, 673–681.

Otta, J. D., & English, H. (1971). Serology and pathology of *Pseudomonas syringae*. *Phytopathology, 61*, 443–452.

Peix, A., Ramirez-Bahena, M. H., & Velazquez, E. (2009). Historical evolution and current status of the taxonomy of genus *Pseudomonas*. *Infection, Genetics and Evolution, 9*, 1132–1147.

Qing, C., Pengfei, Q., Renlin, X., Tambong, J. T., Djama, Z. R., & Wei, L. (2011). Comparison of three typing methods for evaluating the diversity of *Pseudomonas fluorescens* in the rhizosphere. *Journal of Plant Sciences, 6*, 52–65.

Rademaker, J. L. W., & de Bruijn, F. J. (1997). Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In G. Caetano-Anolles & P. M. Gresshoff (Eds.), *DNA markers: protocols* (pp. 151–171). NY, USA, John Wiley & Sons: Application and Overviews. New York.

Renick, L. J., Cogal, A. G., & Sundin, G. W. (2008). Phenotypic and genetic analysis of epiphytic pseudomonas syringae populations from sweet cherry in Michigan. *Plant Disease, 92*, 372–378.

Rouhrazi, K., & Rahimian, H. (2012). Characterization of Iranian grapevine isolates of *Rhizobium* (*Agrobacterium*) spp. *Journal of Plant Pathology, 94*, 555–560.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*, 406–425.

Sarkar, S. F., & Guttman, D. S. (2004). Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology, 70*, 1999–2012.

Schaad, N. W., Jones, J. B., & Chun, W. (2001). *Laboratory guide for the identification of plant pathogenic bacteria* (3th ed.). Paul, Minnesota, USA: Phytopathological Society St.

Scortichini, M., Marchesi, U., Rossi, M. P., & Prospero, P. D. (2002). Bacteria associated with hazelnut (*Corylus avellana* L.) decline are of two groups: *Pseudomonas avellanae* and strains resembling *P. syringae* pv. *syringae*. *Applied and Environmental Microbiology, 68*, 476–484.

Sneath, P. H. A., & Sokal, R. P. (1973). *Numerical taxonomy: the principles and practice of numerical classification*. San Francisco: WH Freeman and Company.

Suslow, T. V., Schroth, M. N., & Isaka, M. (1982). Application of rapid method for gram differentiation of plant pathogenic and saprophytic bacteria without staining. *Phytopathology, 72*, 917–918.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The clustal_X windows interface: flexible strategies for multiple sequence alignement aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Versalovic, J., Koeuth, T., & Lupski, J. R. (1991). Distribution or repetitive DNA– sequences in eubacteria and application of fingerprinting of bacterial genomes. *Nucleic Acids Research, 19*, 6823–6831.

Versalovic, J., Schneider, M., de Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular Cellular Biology, 5*, 25–40.

Vicente, J. G., & Roberts, S. J. (2003). Screening Wild Cherry Micropropagated Plantlets for Resistance to Bacterial Canker. In: "*Pseudomonas syringae* and Related Pathogens". (Eds.): Iacobellis, N. S., *et al*., Biology and Genetic, Kluwer Academic Publishers, Dordrecht, The Netherlands, PP. 467–474.

Vicente, J. G., & Roberts, S. J. (2007). Discrimination of isolates of *Pseudomonas syringae* from sweet and wild cherry using rep-PCR. *European Journal of Plant Pathology, 117*, 383–392.

Watt, P. M., & Hickson, I. D. (1994). Structure and function of type II DNA topoisomerases. *Biochemical Journal, 303*, 681–695.

Weingart, H., & Volksch, B. (1997). Genetic fingerprinting of *Pseudomonas syringae* pathovars using ERIC-, REP-, and IS50-PCR. *Journal of Phytopathology, 145*, 339–345.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A., & Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology, 146*, 2385–2394.