GENETIC CHARACTERIZATION OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAINS FROM STONE FRUITS BASED ON RAPD ANALYSIS IN IRAN

KHAYAMIE S., NIKNEJAD KAZEMPOUR N., RABIE B., SASSANIE S., EBADIE A.A.

Abstract

Bacterial canker is caused by the bacterial pathogen Pseudomonas syringae pv. syringae which is present in Iran. Random amplified polymorphic DNA (RAPD) was used to assess the genetic structure of P. syringae pv. syringae populations. In this research, genetic diversity of 50 P. syringae pv. syringae isolates from stone fruit trees, olive and hazelnut were analyzed. Out of 100 bands, generated with 8 RAPD primers, 72% were polymorphic and scored as molecular markers. Nei-Li similarity coefficients were computed for every pair of strains from data on fragment presence or absence. Relatedness between strains was evaluated by UPGMA clustering. The results of genetic analysis showed that hazelnut isolates, are completely separated from stone fruit isolates. In addition, all of stone fruit isolate were divided in 3 clusters by RAPD primers. This study shows that strains of P. syringae pv. syringae exhibit genetic diversity detectable by RAPD analysis, and that molecular and statistical analysis of RAPD fragments can be used both to distinguish between strains and to determine relatedness between them.

Key words: stone fruit, *Pseudomonas syringae* pv. *syringae,* genetic diversity, RAPD

INTRODUCTION

Pseudomonas syringae pv. *syringae* van Hall, causes canker, leaf spots and necrosis of the bark of cherry, plum, and peach fruit trees. Symptoms caused by this pathogen on leaves, blossoms, and fruit, reported as common disease in Guilan cherry orchards (Niknejad Kazempour et al*.,* 2007). Various molecular techniques have been used to characterize *P. syringae* pv. *syringae* strains. They include Pulsed-Field Gel Electrophoresis (PFGE) (Grothues and Rudolf, 1991), Restriction Fragment Length Polymorphism (RFLP) (Scholz et al., 1994), Random Amplified Polymorphic DNA (RAPD) (Clerc et al., 1998), repetitive-sequence PCR (rep-PCR) (Scortichini et al., 2003), Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA) (Scortichini et al., 2003) and Amplified Fragment Length Polymorphism (AFLP) (Manceau and Brin, 2003). Presently, AFLP analysis can be considered one of the most discriminating genomic methods to distinguish among bacterial strains (Vos et al., 1995). *P. syringae* is a plant-associated bacterial species which has been divided into more than 50 pathovars. Using DNA pairing analysis, Pecknold and Grogan (1973) showed that *P. syringae* is a heterogeneous species. Later, Denny et al. (1988) showed that *P. syringae* pv. *tomato* strains form a genetically homogeneous group that is clearly distinct from *P. syringae* pv. *syringae* strains and that should be considered a genomic species. The random amplified polymorphic DNA (RAPD) technique, first described by Williams et al. (1990), despite some limitations provides a useful approach for evaluating population's genetic differentiation, particularly in the species that are poorly known genetically (Nybom, 2004; Russo, 2007). The problem of low reproducibility in early RAPD analyses can now be overcome through improved laboratory techniques and band scoring procedures (Volis et al., 2001). Another limitation of RAPD markers is their dominant nature (Williams et al., 1990). This peculiarity of RAPDs impedes direct estimations of allele frequency and can bias calculations of population differentiation (Lynch and Walsh, 1998). This problem can be overcome by use of appropriate statistical methods, e.g., analysis of molecular variance (AMOVA) (Excoffier, 2001), which is not influenced by the dominance problem (Dîaz et al., 2001). In this paper, the randomly amplified polymorphic DNA (RAPD) technique were used to determine the relatedness of 50 *P. syringae* pv. *syringae* isolates from different hosts, with the aim to identify potential sources of inoculum arriving in Iran, as well as to explore the applicability of the RAPD technique to reveal the potential genotypic variability of *P. syringae* pv. *syringae*.

MATERIALS AND METHODS

Bacterial strains and media

Fifty isolates of *P. syringae* pv. *syringae* causal bacterium of canker and blossom blight were isolated from diseased leaves or stems of stone fruits (cherry 12 isolates, peach 11 isolates and prune 10 isolates) hazelnut (8 isolates) and olive (9 isolates) in major cultivating areas of Guilan province–Iran, in 2005. One representative isolate has been deposited in the Collection Française de Bactéries Phytopathogèns (CFBP) culture collection. This reference isolate was considered as a typical isolate of *P. syringae* pv. *syringae* (Table 1). The isolates were grown in Luria broth (LB) and nutrient agar (NA). Liquid media cultures were generated from –80°C freezer stocks in

15% glycerol by overnight shaking at 220 rpm in LB at 25°C. The cultures were initiated at an OD600 = 0.05. Media were amended with appropriate antibiotic cyclohexamid (50 µg/ml).

Tab. 1: The number of scored bands (SB), polymorphism information content (PIC) and genetic diversity based on Nei (H) obtained per each primer among the *Pseudomonas syringae* pv. *syringae* isolates

Primer	SB	PIC	(H)
H3	14	0.31	0.32
G13	12	0.37	0.38
H13	15	0.35	0.37
60.2	14	0.41	0.44
70.8	11	0.31	0.27
70.9	11	0.34	0.32
80.7	13	0.35	0.41
80.8	10	0.37	0.38
Average	12.5	0.35	0.36

DNA extraction

For bacterial DNA extraction, the isolates were grown overnight, in nutrient broth (Merck, Darmstadt, Germany), at 26°C and the DNA was extracted as described by Martins et al. (2005). One tube of 1.5 ml was used to centrifuged the cells at 13 000 × g for 5 min and the pellet was suspended in 200 µl Tris 0.1 mol L-1 and added with 200 µl of lysis solution (NaOH 0.2 N and 1% SDS), mixed and deproteinazed with 700 µl of phenol/chloroform/isoamyl alcohol (25 : 24 : 1 v/v/v), homogenized and centrifuged 10 min at 13 000 × g .To precipitate DNA, 700 µl of cold isopropanol was added and spinned, washed in 70% ethanol and centrifuged. Precipitated DNA is dried at room temperature and suspended in 100 µl of water. The method described by Ausubel et al. (1996) was performed comparing 50 strains. The samples from the both methods were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and photographed under UV light. The RAPD was performed according to Ortiz-Herrera et al. (2004).

RAPD analysis

RAPD analysis were carried out in 25 µl volume final, contained 100 ng of genomic DNA, 3 mM $MgCl_2$, 0.4 µM each primer, 1 U of *Taq* DNA polymerase (Promega Corp., Madison, WI), 250 µM each of dCTP, dGTP, dATP and dTTP (Pharmacia) in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100. The thermal cycling profile was as follows: 4 min initial denaturation at 92°C, 40 cycles of 1 min at 94°C, 1 min at 37°C, 3 min at 72°C, followed by a final extension at 72°C for 5 min. PCR products were analysed in 2% agarose gels stained with ethidium bromide and were visualized and photographed using a Gel Documentation System, GDS 8000 (BioRad., California, USA). Negative controls contained water instead of DNA. The primers used were
H3 (5'- AGACGTCCAC -3'),
H13 (5'- GACGCCACAC -3'),
G13 (5'- CTCTCCGCCA -3'),
80.8 (5'- CGCCCTCAGC -3'),
80.7 (5'- GCACGCCGGA-3'),
70.8 (5'- CTGTACCCCC -3'),
70.9 (5'- TGCAGCACCG -3'),
60.2 (5'- GACCGACACG -3'), supplied by CinnaGen Inc. (Belgium).

Cluster analysis

All isolates of *P. syringae* pv. *syringae* were used for cluster analysis based on RAPD results. Each amplification band was scored as 1 (present) or 0 (absent) for all isolates. Average of dissimilarity was calculated using formula established by Nei and Li (1979) with program Multi-Variate Statistical Package (version 3.13 m, Kovach Computing Services). The isolates were clustered by the unweighted average pair group method (UPGMA).

RESULTS

Primers were selected based on reproducibility and polymorphic patterns from this initial screening. Eight primers (H3, H13, G13, 80.8, 80.7, 70.8, 70.9 and 60.2) were selected based on the quantity and quality of their amplified fragments and were then used to survey all strains of *P. syringae* pv. *syringae* (Clerc et al. 1998). These primers allowed the scoring of 100 bands among the amplified fragments of the 50 isolates of *P. syringae* pv. *syringae* of which 72 were unambiguously reproducible and polymorphic (Table 1), suggesting high genetic variability. The number of scored bands (SB) from 10 for the 80.8 primer to 15 for the H13 primer. Primer 60.2 with 0.41, showed the most polymorphism information content (PIC). The number of bands for each primers from 10 to 15. The size of the amplified product ranged from 0315 to 2.23 kb. The results of PCR – RAPD examination with 8 primer for 5 population of P. syringae pv. syringae isolates are shown in Table 2. The Nei's estimate of similarity, based on the number of shared RAPD products, was used to generate a similarity and distance. According to genetic distance, dendrogram showed that five populations divided into two groups. Populations of peach isolates (Psp), cerise isolates (Psc), prune isolates (Pss) and olive isolates (Pso) are in first group, population of hazelnut isolates (Psh) is second group (Table 3). The largest genetic distance, by Nei method used, was between population Psh and Psp, while the shortest one was between Psp and Pss (Figure 1). It is indicated low distance between four populations (Psp, Psc, Pss and Pso) but clustering analysis of individual shows that the each population scattered among the population. This situation indicated high genetic differences among two groups.

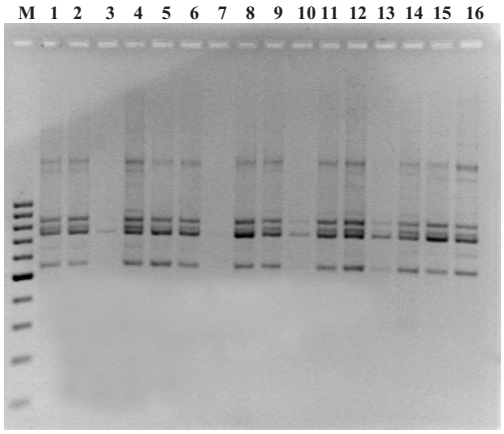
Tab. 2: Information of five populations fragment by different parameters

Population	No. of isolates bacteria	No. of alells (Na)	Effective (Ne)	Nei gene diversity (Nei)
Peach isolate (Psp)	10	1.61 ± 0.49	1.34 ± 0.34	020 ± 0.18
Ceries isolates (Psc)	12	1.86 ± 0.34	1.55 ± 0.35	0.47 ± 0.23
Prune isolates (Pss)	10	1.65 ± 0.47	1.38 ± 0.37	0.22 ± 0.19
Hazelnut isolates (Psh)	8	1.77 ± 0.42	1.53 ± 0.37	0.3 ± 0.19
Olive isolates (Pso)	9	1.71 ± 0.49	1.45 ± 0.37	0.26 ± 0.19
Average	–	2.00	1.62 ± 0.27	036 ± 0.12

Tab. 3: Genetic distance of five populations based on RAPD data

Population	Psp	Psc	Pss	Psh	Pso
Psp	***	0.94	0.95	0.62	0.91
Psc	0.06	***	0.94	0.71	0.89
Pss	0.04	0.05	***	0.62	0.89
Psh	0.46	0.33	0.47	`***	0.65
Pso	0.09	0.11	0.10	0.43	***

Fig. 1: Gels stained with ethidium bromide showing PCR amplification products generated from the *Pseudomonas syringae* pv. *syringae* isolates with primer **H3**, 1, Psp35; 2, Psp28; 3, Psh10; 4, Psc20; 5, Psc17; 6, Psp27; 7, Distille water, 8, Psc96; 9, Psp61; 10, Psh17; 11, Pss82; 12, Pss31; 13, Psh16; 14, Pss26 15, Pss48; 16, Psc101, M= 100 bp DNA marker.

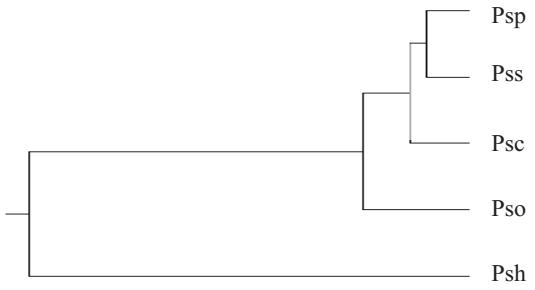


DISCUSSION

P. syringae pv. *syringae* causes canker, leafspots and necrosis of the bark of cherry, plum, and peach fruit trees. The level of RAPD variation and distribution *P. syringae* pv. *syringae* isolates on stone fruit, olive and hazelnut have been investigated. Various primers were tested for efficacy in the RAPD method, and the primers listed in were selected because they gave readily interpretable and reproducible results. All of these primers had GC contents greater than 60%, while the genome GC content of *P. syringae* is 59 to 60 l% (Clerc et al., 1998). The used eight primers were highly informative and revealed an average of 12.5 bands per primer. RAPD analysis made it possible to identify and characterize strains isolated from the same host, and to distinguish between groups of strains isolated from different hosts. *P. syringae* pv. *syringae* strains isolated from *Prunus* hosts in California generated similar genetic profiles in ERIC-PCR whereas most strains of *P. syringae* pv. *syringae* isolated from other hosts generated dissimilar patterns. This suggests a host specialization of the stone fruit strains within the heterogeneous pathovar *syringae* (Little et al., 1998). Hazelnut isolates *P. syringae* pv. *syringae* were separated from stone fruit isolates. The results support that hazelnut and stone fruit isolates of *P. syringae* pv. *syringae* may have different phylogenic origins. In conclusion, RAPD method was suitable for differentiation of *P. syringae* pv. *syringae* isolates. The ability to differentiate between individual isolates may be of potential use in the studies determining the epidemiology of bacteria canker of stone fruit and host-pathogen interactions relating to stone fruit cultivar infection as well as developing improved strategies for

breeding for resistance to *P. syringae* pv. *syringae.* This study shows that RAPD analysis is a useful technique to identify individual strains of *P. syringae* pv. *syringae* and to study their relatedness.

Fig. 2. Dendogram showing relationship between five populations of *P. syringae* pv. *syringae* using UPGM based on Jaccard similarity index from RAPD-PCR data.



REFERENCES

AUSUBEL F.M., BRENT R., KINGSTON E., MOORE D.D., SEIDMANN J.G. (1996): Current Protocols in Molecular Biology. John Wiley & Sons, New York.

CLERC A., MANCEAU C., NESME X. (1998): Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. Applied and Environmental Microbiology, 64: 1180–1187.

DENNY T.P., GILMOUR M.N., SELANDER R.K. (1988): Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. J. Gen. Microbiol., 134: 1949–1960.

DÍAZ V.L., MUÑIZ M., FERRER E. (2001): Random amplified polymorphic DNA and amplified fragment length polymorphism assessment of genetic variation in Nicaraguan populations of Pinus oocarpa. Mol. Eco., 10: 2593–2603.

EXCOFFIER L. (2001): Analysis of population subdivision, In: Balding D.J., Bishop M., Cannings C. (eds.): Handbook of statistical genetics. Chichester (UK): John Wiley & Sons, pp. 271–307.

GROTHUES D., RUDOLPH K. (1991): Macrorestriction analysis of plant pathogenic Pseudomonas species and pathovars. FEMS Microbiology, 79: 83–88.

LITTLE E.L., BOSTOCK R.M., KIRKPATRICK B.C. (1998): Genetic characterization of *Pseudomonas syringae* pv. *syringae* starains from stone fruit in California. Applied and Environmental Microbiology, 64: 3818–3823.

LYNCH M., WALSH B. (1998): Genetics and Analysis of Quantitative Traits. Sinauer Associates, Sunderland, MA.

MANCEAU C., BRIN C. (2003): Pathovars of *Pseudomonas syringae* are structured in genetic populations allowing the selection of specific markers for their detection in plant samples. In: Iacobellis N.S. et al. (eds): *Pseudo-monas syringae* and related pathogens. Kluwer Academic Publishers, Dordrecht, The Netlerlands, pp. 503–512.

MARTINS M.L., ARAÚJO E.F., MANTOVANI H.C., MORAES C.A., VANETTI M.C.D. (2005): Detection of the apr gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology. 102: 203–211.

NEI M., LI W.H. (1979): Mathematical model for studying genetical variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. (USA), 74: 5267–5273.

NIKNEJAD KAZEMPOUR M., JAMIE F., ELAHINIA A.S. (2007): Bacterial Canker Cause By *Pseudomonas syrin-gae* pv. *syringae* on Stone Fruits in Guilan Province of Iran. Int. J. Bio. Biotech., 4 (4): 373–378.

NYBOM H. (2004): Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology, 13 (5): 1143–1155.

ORTIZ-HERRERA M., CHEM P.B., GERÓNIMO-GALLEGOS A. (2004): RAPD-PCR characterization of *Pseudomonas aeruginosa* strains obtained from cystic fibrosis patients. Salud Pública de México, 46: 1–8.

PECKNOLD P.C., GROGAN R.G. (1973): Deoxyribonucleic acid homology groups among phytopathogenic *Pseudomonas* species. Int. J. Syst. Bacteriol., 23: 111–121.

RUSSO E.B. (2007): History of cannabis and its preparations in saga, science and sobriquet. Chemistry and Biodiversity, 4: 2624–2648.

SCHOLZ B.K., JAKOBEK J.L., LINDGREN P.B. (1994): Restriction fragment length polymorphism evidence for genetic homology within a pathovar of *Pseudomonas syringae*. Applied and Environmental Microbiology, 60: 1093–1100.

SCORTICHINI M., MARCHESI U., DETTORI M.T., ROSSI M.P. (2003): Genetic diversity, presence of the *syrB* gene, host preference and virulence of *Pseudomona syringae* pv. *syringae* strains from woody and herbaceous host plants. Plant Pathology, 82: 277–286.

VOS P., HOGERS R., REIJANS M., VAN DE LEE T., HORNES M., FREIJTERS A., POT J., PELEMAN M., KUIPER M., ZABEAU M. (1995): AFLP: a new concept for DNA finger printing. Nucleic Acids Res., 21: 4407–4414.

VOLIS B., YAKUBOV I., SHULGINA D., WARD S., MENDLINGER S. (2005): Distinguishing adaptive from nonadaptive genetic differentiation: comparison of Q_{ST} and F_{ST} at two spatial scales. Heredity, 95: 466–475.

WILLIAMS J.G.K., KUBELIK A.R., LIVAK K.J., RAFALSKI J.A., TINGEY S.V. (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531–6535.

Little E.L., Bostok R.M, Kirkpatrick B.C. (2002) : Genetic Characterization of *Pseudomonas syringae* pv. syringae Strains from Stone Fruits in California. *Applied and Environmental Microbiology,* 64 (10): 3818–3823.

Received for publication on April 4, 2009
Accepted for publication on June 17, 2009

Corresponding author:

Mostafa Niknejad Kazempour
Departments of Plant Pathology,
Biotechnology and Horticulture respectively,
Faculty of Agriculture, University of Guilan,
Rasht – Iran
e-mail: nikkazem@yahoo.fr