International Scholars Journals

African Journal of Food Science Research ISSN 2375-0723 Vol. 9 (3), pp. 001-008, March, 2021. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Identity and genetic diversity of *Pectobacterium* spp., causal agents of potato soft rot in Zanjan, Iran

E. Tavasoli[1], A. R. Marefat[2] and N. Hassanzadeh[1]*

[1]Department of Plant Pathology, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Plant Pathology, Faculty of Agriculture, Zanjan University, Zanjan, Iran.

Accepted 09 October, 2020

Zanjan province is one of the areas of potato production in Iran with more than 6917 ha of cultivation. One of the most important factors to limit potato cultivation is soft rot bacteria belonging to the family Enterobacteriaceae. During the years 2007 to 2008, different potato fields and the most important potato storages in Zanjan province were surveyed. Phenotypic identification of the strains was performed using recommended biochemical and physiological tests. Genetic diversity was determined by BOX-PCR and ERIC-PCR. Also all of the strains were identified with specific primers (Y_1, Y_2 and ECA1f, ECA2r). The strains belonged to "carotovora" group and identified as *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium atrosepticum*. Although there were some atypical characteristics among the strains, but a very close relationship were found between DNA fingerprints and geographical origins of isolates.

Key words: *Pectobacterium*, Soft rot, Black-leg, BOX-PCR, ERIC-PCR

INTRODUCTION

Potato is the fifth most important food crop worldwide. Likewise, Iran is one of the most important potato producing countries in Asia and Oceania. Zanjan province, located in North West of Iran, is one of the areas of potato production in Iran with more than 6917 ha of cultivation. The soft rot erwinias in the Enterobacteriaceae are economically important, because they cause serious damage worldwide on a wide variety of plants (Perombelon and Kelman, 1980).

Pectobacterium carotovorum subsp. *carotovorum* and *Dickeya chrysanthemi* have a wide host range, infecting crops mainly in tropical and subtropical regions, whereas *P. atrosepticum* is restricted almost exclusively to potato in temperate regions, causing soft rot of tubers and blackleg of stems (Perombelon, 1992). Different studies have been done for recognition of these bacteria on potato in some parts of Iran, which were based on physiological and biochemical tests as well as the comparison of protein electrophoretic patterns that in most cases did not lead to a distinct group differentiation (Bahar and Danesh, 1986; Zohoor et al., 1998). One of the tools in studying the genetic diversity of bacteria including potato soft rot Pectobacteria is rep-PCR (Rademaker and de Bruijn, 1997; Xiu et al., 2006). To perform this, many potato soft rot bacteria were isolated from both farms and stores of Zanjan province and their genetic diversity were estimated.

MATERIALS AND METHODS

Sample handling and isolate collection

During the years 2007 to 2008, different potato fields and the most important potato storages in Zanjan province (Abhar, Ijrood, Khodabandeh and Khoramdareh) were surveyed and sufficient samples were collected from plants suffering from soft rot disease. Nutrient agar, King's B agar and Eosin methylene blue agar media were used to isolate the suspected bacteria (Schaad et al., 2001). The type strain of *Erwinia carotovora* 5702 was obtained from the International Collection of Micro-organisms from Plants (ICMP), Auckland, New Zealand.

*Corresponding author. E-mail: hasanzadehr@yahoo.com.

Biochemical and physiological tests

All strains were tested for gram reaction using the KOH test, potato soft rot, oxidase reaction, catalase reaction, gelatin hydrolysis, production of gas from glucose, production of reducing substances from sucrose, growth in 5 and 6% NaCl, ability to grow at 37ºC and phosphatase (Schaad et al., 2001), fermentative metabolism (Hugh and Leifson, 1953), lecithinase (Fahy and Hayward, 1983), production of indole (Dye, 1968), and sensitivity to erythromycin (Psallidas, 1993). Additional biochemical tests were starch hydrolysis, esculin hydrolysis, nitrate reduction, arginine dihydrolase, action on litmus milk, urease production, H_2S production from cysteine (Schaad et al., 2001), Tween-80 hydrolysis (Misaghi and Grogan, 1969), casein hydrolysis (Fahy and Hayward, 1983), 3-ketolactose production (Cowan, 1974), methyl red reaction and acetoin production (Dye, 1968) and acid production from carbohydrates utilized as a source of carbon (Schaad et al., 2001).

Preparation of DNA and Rep-PCR genomic fingerprinting

Genomic DNA was extracted using the whole cell alkaline lysis method (Rademaker and de Bruijn, 1997). The DNA was stored at -20°C until it was required. All strains were subjected to BOX-PCR using the BOX A1R primer (5´-CTA CGG CAA GGC GAC GCT GAC G-3´) (Martin et al., 1992). ERIC-PCR was performed for 10 selected strains from different groups of BOX-PCR by using the ERIC 1R primer (5´-TGT AAG CTC CTG GGG ATT CAC-3´) and ERIC 2 primer (5´-AAG TAA GTG ACT GGG GTG AGC G-3´) (Versalovic et al., 1991). The method developed by Rademaker and de Bruijn (1997) was performed with slight modification in cycling conditions. The PCR master mix (25 µl) containing: 1 µl DNA, 0.3 µl Taq DNA polymerase (0.1 U), 2.5 µl 10 × PCR buffer, 0.5 µl dNTPs (25 mM), 1 µl $MgCl_2$ (25 mM), 1.1 µl of each primer (10 pmol). DNA amplification was performed on a thermocycler (Corbett, Germany) under the following conditions: 4 min at 94ºC for initial denaturation, 34 cycles of 40 s at 94ºC, 40 s at 50ºC and 1 min at 72ºC, followed by a final elongation step of 10 min at 72ºC. PCR products (6 µl) were separated by gel electrophoresis in 1.5% agarose gels in TBE buffer. Following staining with ethidium bromide, the gels were viewed and photographed under UV Transilluminator. A 1-kb marker (Fermentas, Germany) was included on every gel. Captured photographs were subjected to the Gel-Pro® Analyzer (Media Cybernetics, MD, USA) computer program. Bands were scored in binary form, 1 and 0, indicating the presence and absence of a band, respectively. A similarity matrix was obtained using the Jaccard coefficient and the software package NTSYS-pc (version 2.02K, Applied Biostatistics, Inc., NY, USA). To determine the relationship among the strains, cluster analysis was performed with UPGMA (unweighted pair-group method, using arithmetic averages) in the SAHN program of the NTSYS-pc software. Dendrograms were generated for the BOX and ERIC binary matrixes individually.

Identification and detection with specific primers

Primers Y_1 (5´- TTA CCG GAC GCC GAG CTG TGG CGT-3') and Y_2 (5'-CAG GAA GAT GTC GTT ATC GCG AGT-3') selected from the pectate lyase-encoding *pel* gene sequences of *E. carotovora* (Darrasse et al., 1994), were used. A specific PCR assay for *P. atrosepticum* was performed using primers ECA1f (5'-CGG CAT CAT AAA AAC ACG-3') and ECA2r (5'-GCA CAC TTC ATC CAG CGA-3') following the protocol of De Boer and Ward (1995), with slight modifications. PCR master mix (25 µl) with Y_1 and Y_2 primers containing: 2.5 µl DNA, 0.1 U Taq DNA polymerase, 2.5 µl 10 × PCR buffer, 2 µl dNTPs (1 Mmol), 1.5 µl $MgCl_2$ (25 mmol), 0.1 µmol of each primer. DNA amplification was performed on a icycler (BIO-RAD) under the following conditions: 5 min at 94ºC for initial denaturation, 34 cycles of 30 s at 94ºC, 45 s at 55ºC and 45 s at 72ºC, followed by a final elongation step of 7 min at 72ºC. PCR reaction mix in final reaction volumes of 25 µl was prepared containing 2.5 µl DNA, 0.5 U Taq DNA polymerase, 2.5 µl 10 × PCR buffer, 100 µM each of dNTPs, 2 mM $MgCl_2$, 0.5 µM of each primer. DNA amplification was performed on a icycler (BIO-RAD) under the following conditions: 5 min at 95ºC for initial denaturation, 40 cycles of 30 s at 94ºC, 45 s at 62ºC and 45 s at 72ºC, followed by a final elongation step of 8 min at 72ºC. PCR products (6 µl) were separated by gel electrophoresis in 1.5% agarose gels in TBE buffer. Following staining with ethidium bromide, the gels were viewed and photographed under UV transilluminator.

RESULTS

Biochemical and physiological tests

On the basis of biochemical profiles, 67 and 2 strains were identified as *P. carotovorum* and *P. atrosepticum*, respectively. All of them were gram negative, facultatively anaerobic, oxidase negative, catalase positive, non-fluorescent on King´s B (KB) medium and showed soft rot symptoms on slices of potato. All strains grew at 37ºC and in 5% NaCl. Gelatin hydrolysis and lecithinase were negative. Some of them produced gas from glucose (22.3%), indole (59.2%) and phosphatase (41.8%). 10.4% of strains showed sensitivity to erythromycin. 3% were able reduce substances from sucrose and 94% were able to tolerate 6% NaCl. Thus these strains were identified as the "carotovora" group belonging to the family Enterobacteriaceae (Tables 1 and 2). Results of complementary tests on 22 strains (selected by results of BOX-PCR), showed that no strains produced urease, 3-ketolactose and acetoin, also no strains were able to hydrolyse Tween-80. They hydrolyzed starch (22.7%), casein (27.3%) and esculin (77.3%). Litmus milk reaction was acid (86.4%) and arginine dihydrolase was positive (72.7%). All strains could reduce nitrate and produced H_2S from cysteine. 50% of strains were positive in methyl red reaction. All of them produced acid from arabitol, arabinose, trehalose, rhamnose, ribose, cellobiose, glucose, fructose, sucrose or lactose, but none produce acid from insulin. In addition they could produce acid from α-methyl-D-glucoside and raffinose (81.8%), maltose (86.4%), sorbitol and myo-inositol (77.3%), melibiose and palatinose (68.2%). All of them utilized citrate and 86.4% utilized malonate. No strains utilized D-tartrate. Results showed a high variability among strains. Results also revealed that *P. carotovorum* was the main pathogen and *D. chrysanthemi* was absent from the strains tested.

Rep-PCR genomic fingerprinting

PCR using the BOX primer gave genomic fingerprints with 22 bands, ranging from about 250 to 3000 bp. Results revealed two main clusters among the pathogen strains and their similarity value, based on UPGMA, was

Table 1. Bacterial strains used in this study.

Strain	Location of origon	Year isolated
Z1, Z2, Z3, Z4	Kheirabad	2007
Z5, Z6, Z7, Z8, Z9	Khoramdareh	2007
Z10, Z11, Z12, Z13	Abhar	2007
Z14, Z15, Z16, Z17, Z18	Gonbad soltanieh	2007
Z19, Z20, Z21, Z22, Z23, Z24	Khodabandeh	2007
I1, I2, I3, I4, I5, I6, I7, I8, I9, I10, I11, I12, I13, I14, I15, I16, I17, I18, I19, I20, I21	Ijrood	2007
E2, E15, E26, E29, E24, E25, E32, E34, E35, E41, E40, E37	Ijrood	2008
B5, D8, D9, D10, D20, D22, D25, D34, D17, D24	Zanjan	2008

Table 2. Phenotypic characteristics of the strains of *Pectobacterium* isolated from potato in comparison with standard isolate.

Test	Results		
	Number of strains positive or negative/number of strains tested	Positive strains (%)	*P. carotovorum* 5702
Fermentative growth	+(67/67)	100	+
Gram reaction	-(67/67)	0	-
Potato soft rot	+(67/67)	100	+
Oxidase	-(67/67)	0	-
Catalase	+(67/67)	100	+
Lecithinase	-(67/67)	0	-
Gelatin hydrolysis	-(67/67)	0	+
Gas from glucose	+(52/67)	77.6	-
Reducing substances from sucrose	-(65/67)	3	-
Growth in 5% NaCl	+(67/67)	100	+
Growth in 6% NaCl	+(63/67)	94	+
Growth at 37ºC	+(67/67)	100	+
Production of Indole	+(39/67)	58.2	-
Sensivity to erythromycin	-(60/67)	10.4	+
Phosphatase	-(39/67)	41.8	+
Starch hydrolysis	-(17/22)	22.8	-
Tween-80 hydrolysis	-(22/22)	0	-
Casein hydrolysis	-(16/22)	27.3	+
Urease production	-(22/22)	0	-
Methyl red reaction	+(11/22)	50	+
Acetoin production	-(22/22)	0	-
Esculin hydrolysis	+(17/22)	77.3	+
3-ketolactose production	-(22/22)	0	-
Nitrate reduction	+(22/22)	100	+
Arginine dihydrolase	+(16/22)	72.8	-
H_2S from cysteine	+(22/22)	100	+
	Action on Litmus milk:		
Acid reaction	(19/22)	86.4	+
Alkaline reaction	(3/22)	13.6	-
	Acid production from:		
Glucose	+(22/22)	100	+
Fructose	+(22/22)	100	+
Sucrose	+(22/22)	100	+

Table 2. Contd.

α-methyl-D-glucoside	+(18/22)	81.8	+
Sorbitol	+(17/22)	77.3	+
Melibiose	+(15/22)	68.2	+
Raffinose	+(18/22)	81.8	+
Arabitol	+(22/22)	100	+
Lactose	+(22/22)	100	+
Trehalose	+(22/22)	100	+
Rhamnose	+(22/22)	100	+
Maltose	+(19/22)	86.4	+
Palatinose	+(15/22)	68.2	-
Ribose	+(22/22)	100	+
Inulin	-(22/22)	0	-
Cellobiose	+(22/22)	100	+
Myo-inositol	+(17/22)	77.3	+
Arabinose	+(22/22)	100	+
Utilization of:			
Citrate	+(22/22)	100	+
D-tartrate	-(22/22)	0	-
Malonate	+(19/22)	86.4	+

+, positive reaction; -, negative reaction.

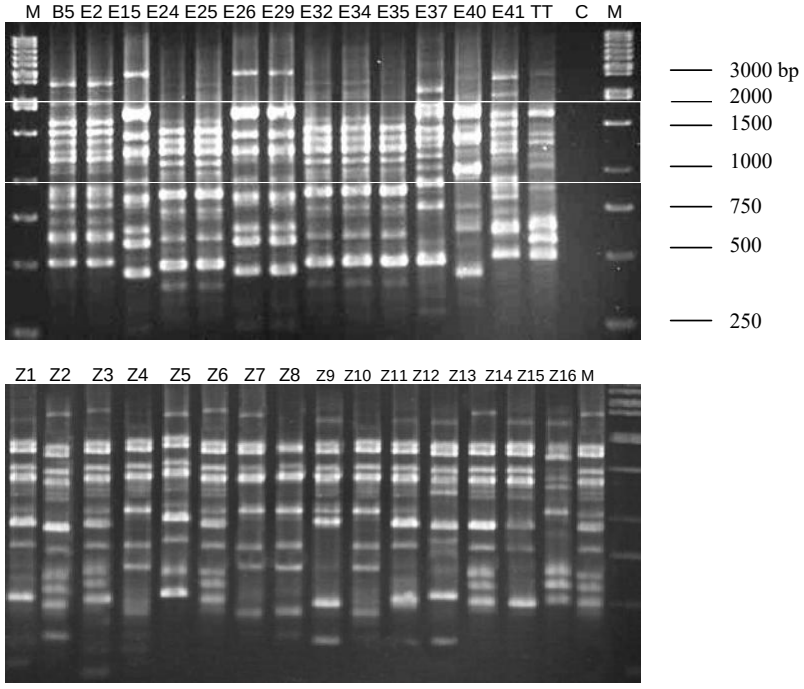

Figure 1. Genomic DNA fingerprinting patterns from strains of *Pectobacterium* isolated from potato, generated by BOX-PCR**.** M: Marker (1-kb); C: Control (without DNA); TT: *P. carotovorum* 5702.

about 51.5%. The first group was divided into two subgroups at 65.5% similarity. The second group was also divided into two subgroups at 75.5% similarity. This protocol revealed 18 genotypes within the pathogen (Figures 1 and 2). Based on BOX-PCR results, 10 strains selected from these groups, were further analyzed with

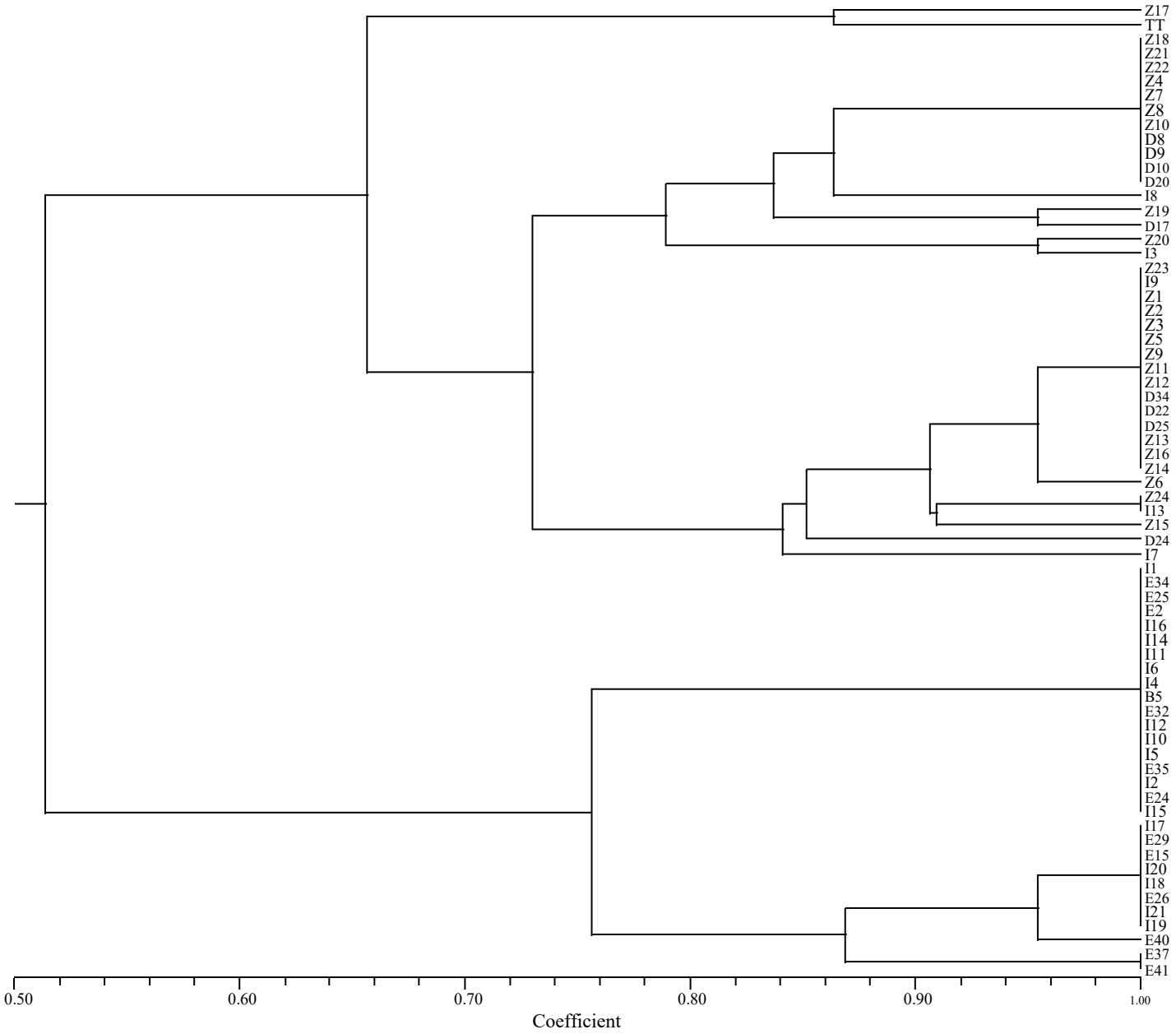

Figure 2. Similarity dendrogram of *Pectobacterium* strains based on BOX-PCR.

ERIC-PCR using the ERIC 1R primer and ERIC 2 primer. Genomic fingerprints in ERIC-PCR showed 28 bands, ranging from about 250 to 3500 bp including 9 genotypes. Based on UPGMA, the similarity value bet-ween the two main clusters was about 46.5%. The first group divided into two subgroups at 50% similarity and the second group showed one genotype (Figures 3 and 4).

Identification and detection with specific primers

All strains from the Zanjan province yielded a 434 bp DNA fragment in PCR with the Y_1 and Y_2 primers (Figure 5) and only two strains (E2 and D24) yielded a 690 bp DNA fragment in PCR with the ECA1f and ECA2r primers (Figure 6). Therefore all of the studied strains were identified as *Pectobacterium carotovorum* and only two strains as *P. atrosepticum***.**

DISCUSSION

In this research, the characteristics of pectolytic *Erwinias* of potato in the Zanjan province and their genetic diversity were determined both biochemically and by the

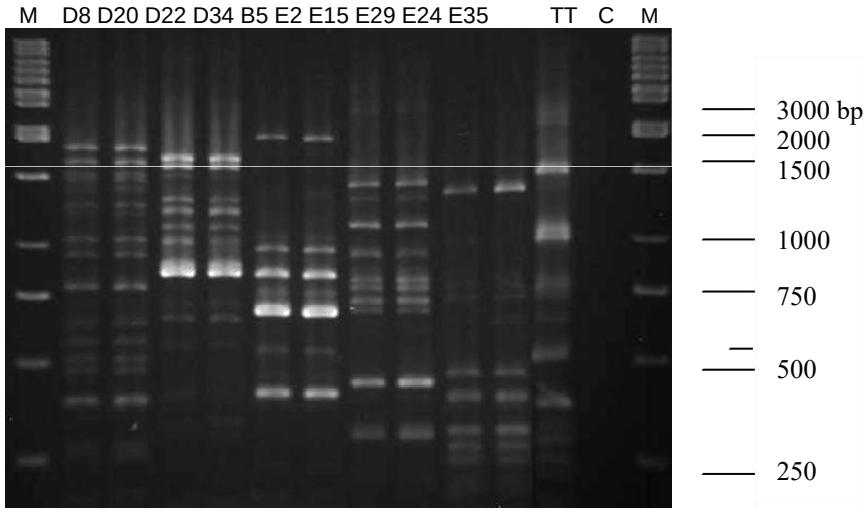

Figure 3. Genomic DNA fingerprinting patterns from representative strains of *Pectobacterium* isolated from potato, generated by ERIC-PCR. M: Marker (1-kb); C: Control (without DNA), and TT: *P. carotovorum* 5702

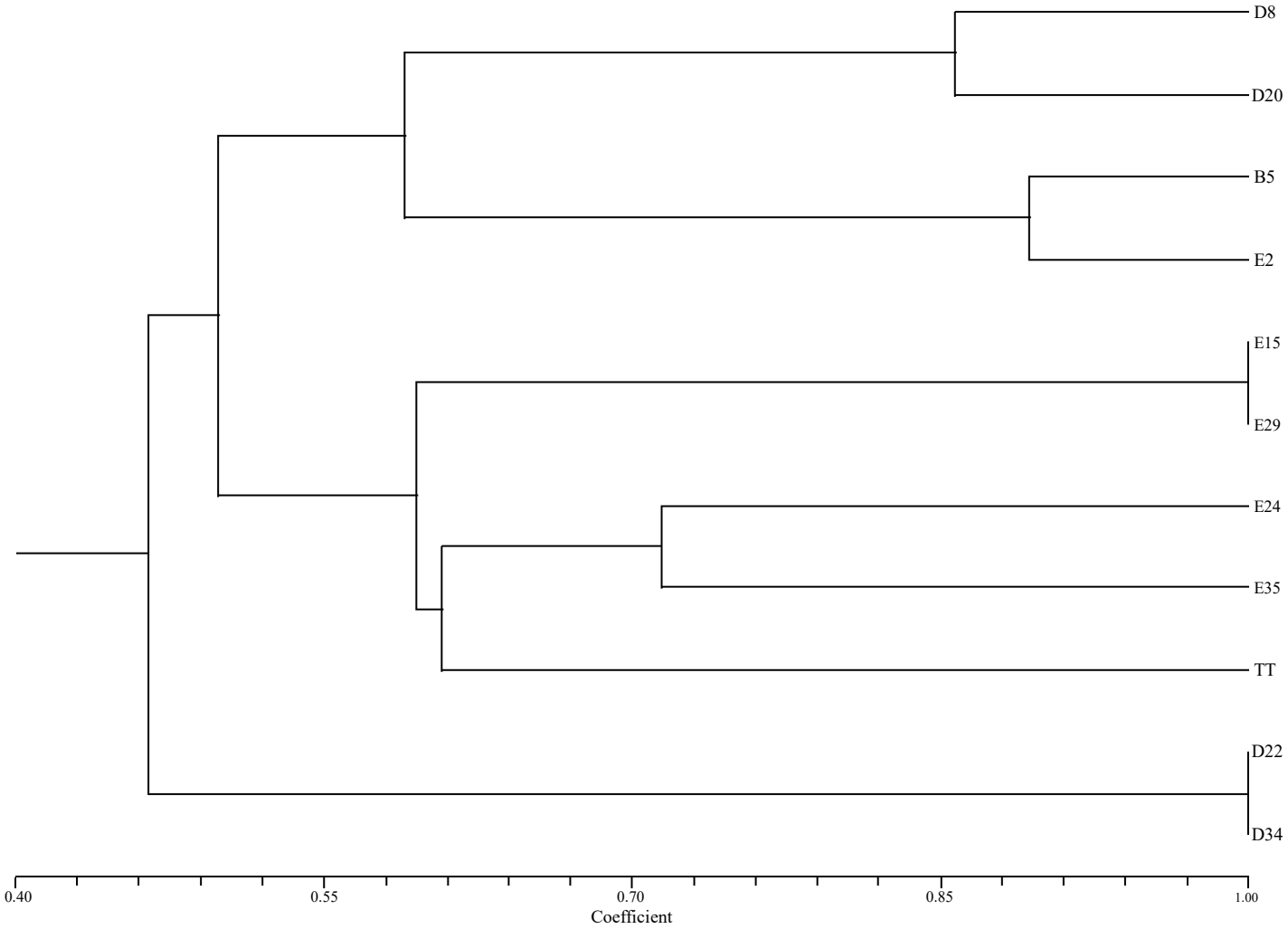

Figure 4. Similarity dendrogram of *Pectobacterium* strains based on ERIC-PCR.

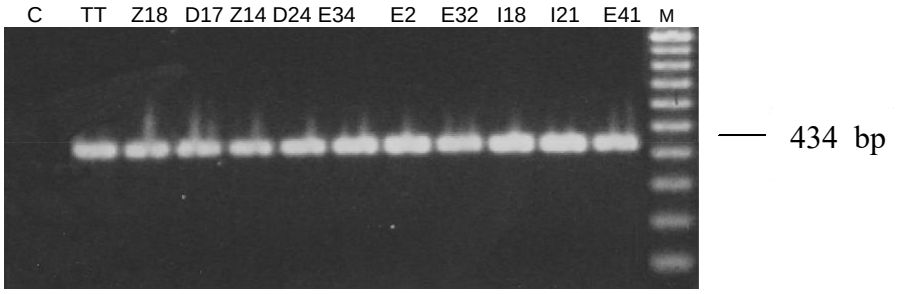

Figure 5. Amplification of a specific 434 bp band in 10 strains generated using Y_1 and Y_2 primers. C: Water control; M: Marker (100 bp); TT: *P. carotovorum* 5702.

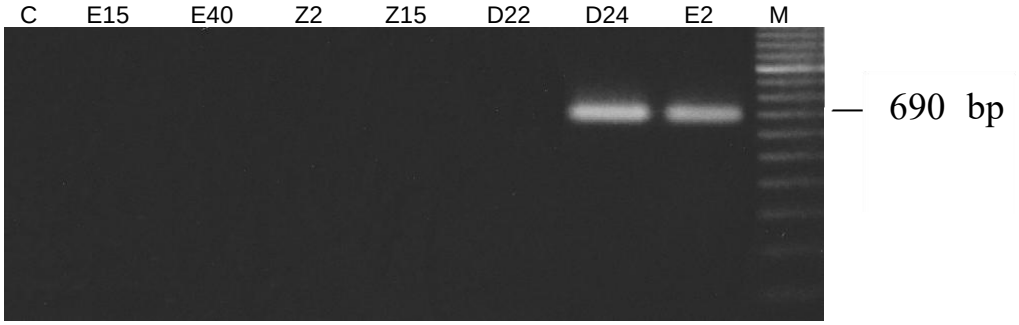

Figure 6. Amplification of a specific 690 bp band in 7 strains, generated using ECA1f and ECA2r primers. C: Water control; M: Marker (100 bp).

rep-PCR technique. The specific primers were used for the recognition of these pathogens.

There was a considerable variation in phenotypic traits and gene electrophoretic clusters among the strains. In this case, 97% of strains were similar to *P. carotovorum* and only 3% were similar to *P. atrosepticum.* Properties of a few strains did not correspond with defined characteristics of authentic species and subspecies of *Pectobacterium*. These findings agree with Louw's justification on Rep-PCR and its high efficiency towards discrimination of pathogen population diversity (Louws et al., 1994).

In this study, some correlations between biochemical characteristic and BOX-PCR based genetic fingerprints among some of the strains were observed. Thus strains related to a defined genetic group with the same genotype, showed the same phenotype characteristics and vice versa. An exceptional example is the D24 strain, which was phenotypically different from other strains showed 85% similarity in genotype to those of the same group.

Comparing the results of genetic fingerprint BOX-PCR and ERIC-PCR, some similarities and differences were observed in their classification. In both cases, strains were placed in two main fingerprinting groups. In none of the current classifications has complete similarity between the standard strain and the examined one been reported. Thus there was no complete correspondence between the BOX-PCR results and ERIC-PCR and even those strains which showed the same genotype in BOX-PCR showed different genotypes in ERIC-PCR.

Geographically, the group created by BOX-PCR was closely related to their sample collection areas. Because of this, most of strains of a single area were placed in one of the main groups. In ERIC-PCR, although only a limited number of strains were compared, these correlations were defined. Linkage between rep-PCR results and the geographic origin of bacterial strains has been recognized in various studies (Scortichini et al., 2001; Mkandawire et al., 2004). Louws et al. (1994) believe that one of the important reasons for this phenomenon is that the selection for one geographically suitable area can have influence on the genetic map of bacterium and also dispersion of these repetitive units in the genome of bacterium. This work supports this idea and in some cases artifacts, these relations were observed. No doubt rep-PCR is a reliable tool for epidemiological studies of diseases and one can use the information as a device for detection of pathogens. Despite this, more recent work with ISSR-PCR of bacteria such as *Clavibacter michiganensis* subsp. *michiganesisis* proved the greater sensitivity, specificity and reliability of this technique as another helpful informative tool in epidemiological studies (Baysal et al., 2011).

Examining strains in Zanjan province, with special primers of Y_1/Y_2, specialized for *P. carotovorum*

(Darrasse et al., 1994) and ECA1f, ECA2r, specialized for *P. atrosepticum* (De Boer and Ward, 1995), the main pathogen of potato soft rot in the Zanjan province is *P. carotovorum* and in some cases *P. atrosepticum*. Failing to isolate *D. chrysanthemi* is not strange in the province, since this bacterium was reported in delimit potato cultivation areas of the Hamedan province. Besides this, the weather of Zanjan province is cold enough to limit the growth of the bacterium (Agrios, 1988).

REFERENCES

Agrios GN (1988). Plant Pathology. 3rd ed. Academic Press, New York. p. 803.

Bahar M, Danesh D (1986). Occurrence of potato blackleg in Isfahan. Proceedings 8th Plant Protection Congress of Iran. p. 105.

Baysal Ö, Mercati F, Ikten H, Yıldız RÇ, Carimi F, AysanY, Teixeira da Silva JA (2011). *Clavibacter michiganensis* subsp. *michiganesis*: Tracking strains using their genetic differentiations by ISSR markers in Southern Turkey. Physiol. Mol. Plant Pathol., 75: 113-119.

Cowan ST (1974). Cowan and Steel, manual for the identific of medical bacteria, 2nd. Combridge University Press, Cambridge.

Darrasse A, Priou S, Kotoujansky A, Bertheau Y (1994). PCR and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. App. Environ. Microbiol., 60: 1437-1443.

De Boer SH, Ward LJ (1995). PCR detection of *Erwinia carotovora* subsp. atroseptica associated with potato tissue. Phytopathol., 85: 854-858.

Dye DW (1968). A taxonomic study of the genus *Erwinia*. I. The `amylovora' group. New Zealand J. Sci., 11: 590-607.

Fahy PC, Hayward AC (1983). Media and methods for isolation and diagnostic test. In: Fahy, P. C., Persley, G. J. (eds.) Plant Bacterial Disease A Diagnostic Guide. pp. 337-378. Academic Press. Sidney, Australia.

Hugh R, Leifson E (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol., 66: 24-26.

Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. App. Environ. Microbiol., 60: 2286-2295.

Martin B, Humbert O, Camara M, Guenzi E, Walker J, Mitchell T, Andrew P, Prudhomme M, Alloing G, Hakenbeck R (1992). A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res., 20: 3479-3483.

Misaghi L, Grogan RG (1969). Nutritional and biochemical comparison of plant pathogenic and saprophytic fluorescent Pseudomonads. Phytopathol.. 59: 1436-1450.

Mkandawire ABC, Mabagala RB, Guzman P, Gepts P, Gilbertson RL (2004). Genetic diversity and pathogenic variation of common blight bacteria (*Xanthomonas campestris* pv*. phaseoli and X. campestris* pv*. phaseoli* var. *fuscans*) suggests pathogen coevolution with the common bean. Phytopathology, 94: 593-603.

Perombelon MCM (1992). Potato blackleg: epidemiology, host–pathogen interaction and control. Netherlands J. Plant Pathol., 98(Suppl. 2): 135-146.

Perombelon MCM, Kelman A (1980). Ecology of the soft rot erwinias. Ann. Rev. Phytopathol., 18: 361-387.

Psallidas PG (1993). *Pseudomonas syringae* pv. *avellanae* pathovar nov., the bacterium causing canker disease on *Corylus avellanae*. Plant Pathol., 42: 358-363.

Rademaker JLW, de Bruijn FJ (1997). Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In: Caetano-Anolles G, Gresshoff PM, eds. DNA Markers: Protocols, Application and Overviews. New York, USA: John Wiley & Sons, pp. 151-171.

Schaad NW, Jones JB, Chun W (2001). Laboratory guide for identification of plant pathogenic bacteria. Third Ed, APS Press, St. Paul, Minnesota, USA., p. 373.

Scortichini M, Marchesi U, Di Prospero P (2001). Genetic diversity of *Xanthomonas arboricola* pv*. juglandis* (synonyms: *X. campestris* pv*. juglandis; X. juglandis* pv*. juglandis*) strains from different geographical areas shown by repetitive polymerase chain reaction genomic fingerprinting. J. Phytopathol., 149: 325-332.

Versalovic J, Koeuth T, Lupski JR (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acid Res., 19: 6823-6831.

Xiu JH, Ji GH, Wang M, Yang YL, Li CY (2006). Molecular identification and genetic diversity in Konnyaku's soft rot bacteria. Acta. Microbiologica Sinica, 46(4): 522-525.

Zohoor SM, Rahimian H, Banihashemi Z (1998). Identity of soft rot *Erwinia* as infecting potato in Fars. Proceedings 13th Plant Protection Congress of Iran, p 171.