Genetic Diversity and Pathogenicity of *Xanthomonas axonopodis* Strains Inducing Citrus Canker Disease in Iran and South Korea

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Abstract For the first time in 1989 citrus bacterial canker disease has been seen on *Citrus aurantiifolia* in southern Iran. A total of 43 strains from affected citrus trees, ten strains from South Korea and representative from all known five pathotypes of *Xanthomonas axonopodis* pathogenic on citrus trees were used in this study. Isolated strains from Iran were indistinguishable by phenotypic, FAMES, and SDS-PAGE analyses but showed different host range. First group were pathogenic on all tested citrus seedlings including *C. aurantiifolia*, *C. limettioides*, *C. limon*, *C. jambhiri*, *Poncirus trifoliata* X *C. paradisi*, *C. aurantium*, *C. paradise*, *C. medica*, *P. trifoliata*, *C. grandis*, *C. sinensis*, *C. reticulata* and *C. sinensis* X *P. trifoliata*. Pathogenicity of the second group were limited to *C. aurantiifolia*, *C. limettioides*, *C. limon*, *C. jambhiri*, *P. trifoliata* X *C. paradisi*, and *C. aurantium*. Among the strains studied by AFLP fingerprinting six clusters were found. These clusters were: (1) strains of pathotype C; (2) strains of pathotypes B and D; (3) strains of pathotype A together with the main group of the Iranian strains; (4) strains isolated from Korea; (5) strains of pathotype E; and (6) seven strains from Iran which made a completely separate cluster. Strains from pathotypes B and D could not be differentiated by AFLP. The tested Iranian strains belong to the two different groups and strains from Korea grouped as a subcluster from main cluster of Iranian strains belong to the pathotype A.

Keywords Citrus canker · *Xanthomonas axonopodis* pv. *citri* · AFLP · Iran. South Korea

Introduction

Citrus bacterial canker disease which seriously threatens citrus crops is caused by *Xanthomonas axonopodis* Starr and Garces emend Vauterin et al. pv. *citri* (Hasse) Dye [syn. *Xanthomonas campestris* pv. *citri* (Hasse) Dye]. Symptoms of the disease include necrotic lesions on leaves, twigs and fruits which in severe infections can cause leaf abscission, twig dieback and premature fruit drop [6, 19, 21, 22]. At least five forms or pathotypes of the bacterium have been identified based on host range, symptomatology, phage typing, DNA fingerprinting and geographical distribution. These pathotypes named as forms A or *X. a.* pv. *citri*, B, C, D or *X. a.* pv. *aurantifolii* and E or *X. a.* pv. *citrumelo* [23, 24]. Of these pathotypes form A which causes true or Asiatic canker disease is the most devastating form and can infect most commercial citrus varieties and citrus relatives [19, 21, 23]. This disease occurs in at least 30 tropical and subtropical countries.

Extensive DNA fingerprinting in bacterial taxonomy has shown that the distribution of restriction endonucleases cleavage site in a bacterial genome is unique, stable, clonally inherited and can be directly observed [10, 15]. Based on this property, restriction fragment length polymorphism (RFLP) of bacterial citrus canker strains has clearly shown that pathotypes B, C and D are clonally related but highly distinct from those of groups A and E. Furthermore these studies have revealed that strains from groups B and D are indistinguishable from each other [3, 4, 7, 9].

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Many studies have shown that amplified fragment length polymorphism (AFLP) is a powerful and high resolution DNA fingerprinting method which can be used to identify bacterial strains at a subgeneric level [2, 10, 11, 15]. Rademaker et al. [17] compared AFLP, rep-PCR and DNA–DNA homology for the strains of genus *Xanthomonas* and observed high correlation, suggesting that genomic fingerprinting techniques truly reveal genotypic and phylogenetic relationships of organisms. Gonzalez et al. [5] using AFLP distinguished pathogenic and nonpathogenic strains of *X. axonopodis* pv. *manihotis* from each other and also from other *Xanthomonas* strains. Restrepo et al. [18] characterized genetic structure and population dynamics of *X. axonopodis* pv. *manihotis* in Colombia from 1995 to

1999 and identified 45 RFLP types or haplotypes. Kolliker et al. [14] studied genetic diversity of *X. translucens* pv. *graminis* by AFLP and found two distinct clusters. Simoes et al. [20] to a better understanding of the genetic and phylogenetic relationships of *Xanthomonas* species used PCR-RFLP analyses.

Citrus bacterial canker disease in Iran was first reported in 1989 from Kerman province in southern Iran but its pathotype(s) was(were)not identified [1] Isolated strains from infected areas in southern Iran were indistinguishable by phenotypic, FAMES and SDS-PAGE [12, 13].

More than 40 strains from Iran, ten strains from South Korea and representative from all known five pathotypes of *X. axonopodis* pathogenic on citrus trees were used in this

Table 1 Strains of *Xanthomonas axonopodis* pathogenic on citrus trees, used in this study

Code no.	LMG or R-no.	Host	Place of isolation	Pathogenicity group	AFLP group
1	LMG-682 (T) ^a	<i>Citrus lemon</i>	New Zealand	A	3
2	LMG-9176	<i>C. aurantifolia</i>	Brazil	A	3
3	LMG-9652	Tangerine	Korea	A	3
4	LMG-9659	<i>Citrus</i> sp.	Guam	A	3
5	LMG-9183	<i>C. lemon</i>	Argentina	B	2
6	LMG-9179	<i>C. lemon</i>	Argentina	B	2
7	LMG-8655	<i>C. aurantifolia</i>	Brazil	C	1
8	LMG-8656	<i>C. aurantifolia</i>	Brazil	C	1
9	LMG-9181	<i>C. aurantifolia</i>	Brazil	C	1
10	LMG-9654	<i>C. aurantifolia</i>	Brazil	C	1
11	LMG-9658	<i>C. aurantifolia</i>	Brazil	C	1
12	LMG-9182	<i>C. aurantifolia</i>	Mexico	D	2
13	LMG-9160	Citrumelo	Florida	E	5
14	LMG-9163	<i>C. paradisi</i>	Florida	E	5
15	R-4867,4868,4869,4870	<i>C. aurantifolia</i>	Iran	A	3
	4876, 4877, 4878, 4880	<i>C. aurantifolia</i>	Iran	A	3
	4891, 4893	<i>C. aurantifolia</i>	Iran	A	3
	4904, 4906, 4916, 4921,	<i>C. limettioides</i>	Iran	A	3
	5226, 5228a, 5228b,	<i>C. paradisi</i>	Iran	A	3
	5229, 5235, 5238	<i>C. paradisi</i>	Iran	A	3
	5240a, 5240b, 5242,	<i>C. sinensis</i>	Iran	A	3
	5421, 5422	<i>C. sinensis</i>	Iran	A	3
	5423, 5424, 5425,	<i>C. limettioides</i>	Iran	A	3
	5426, 5427	<i>C. limettioides</i>	Iran	A	3
	5433, 5434, 5435	<i>C. aurantifolia</i>	Iran	A	3
	5436, 5439, 5442	<i>C. aurantifolia</i>	Iran	A	3
	R-4917,4918,5227,	<i>C. aurantifolia</i>	Iran	F	6
	5234	<i>C. aurantifolia</i>	Iran	F	6
	5430, 5431, 5432	<i>C. limettioides</i>	Iran	F	6
17	R-6208, 6209, 6210,	<i>C. sinensis</i>	Korea	A	4
	6211, 6212, 6213,	<i>C. sinensis</i>	Korea	A	4
	6214,6215, 6216,	<i>C. sinensis</i>	Korea	A	4
	6217	<i>C. sinensis</i>	Korea	A	4

^a Pathotype reference strain

Table 2 Pathogenicity reaction of the citrus pathogenic *X. axonopodis* strains isolated from southern Iran and South Korea

Plant	Pathogenicity of group I	Pathogenicity of group II
<i>C. aurantiifolia</i>	+	+
<i>C. limettioides</i>	+	+
<i>C. limon</i>	+	+
<i>C. jambhiri</i>	+	+
<i>Poncirus trifoliata</i> X <i>C. paradisi</i>	+	(+)
<i>C. aurantium</i>	+	(+)
<i>C. paradise</i>	+	–
<i>C. medica</i>	+	–
<i>P. trifoliata</i>	+	–
<i>C. grandis</i>	+	–
<i>C. sinensis</i>	+	–
<i>C. reticulata</i>	+	–
<i>C. sinensis</i> X <i>P. trifoliata</i>	+	–

+ positive reaction, – negative reaction, (+) weak reaction

study. The main purpose of this study were determination of host range of the collected citrus pathogenic *X. axonopodis* strains and differentiation of them by AFLP in order to better understanding of their relationships.

Materials and Methods

Bacterial Strains and Cultures

The strains studied are listed in Table 1. These included 43 strains from infected citrus trees in southern Iran, ten representative strains of pathotype A from Korea selected among 20 strains which gifted by Dr. Inn-Shik Myung¹ (indicated by R letter) and 14 reference strains from the five pathogenicity groups A, B, C, D and E (indicated by LMG) which were obtained from the culture collection of the Laboratory of Microbiology, University of Gent, Belgium. The reference strains have been described in details elsewhere [23]. Pathogenicity of Iranian strains were determined on citrus species seedling [12]. Bacterial cells were grown on trypticase soy broth agar [3% w/v trypticase soy broth (BBL) supplemented with 1.5% bacto agar (Difco)] at 28°C.

Pathogenicity Test

Pathogenicity of tested strains were determined by method of Graham et al. 1990 [7] on seedling leaves of citrus species including *Citrus aurantiifolia*, *C. limettioides*,

C. limon, *C. jambhiri*, *Poncirus trifoliata* X *C. paradisi*, *C. aurantium*, *C. paradise*, *C. medica*, *P. trifoliata*, *C. grandis*, *C. sinensis*, *C. reticulata* and *C. sinensis* X *P. trifoliata*. In brief aqueous cell suspensions (approximately 108 CFU ml⁻¹ determined by spectrophotometer) of bacterial cells were prepared from fresh cultures. Young leaves of citrus seedlings were punctured with needle and they were inoculated with a drop of bacterial suspension. Inoculated seedlings were covered with plastic bags for 48 h and put in greenhouse with 28°C until symptoms appeared. Normally distinct citrus bacterial canker symptoms under favorite condition is appear after 2 weeks but results of pathogenicity test were monitored 45 days after inoculation. The causative agent of citrus bacterial canker re-isolated from leaves spots.

Preparation of Samples for AFLP Fingerprinting

Total genomic DNA from bacterial cells was extracted and purified according to Pitcher et al. [16]. Enzyme reactions and PCR were conducted in sterilized Mili Q water. Basically all procedures and materials concerning AFLP were as described by Janssen et al. [10]. Sequences of the used adaptors and primers were as follows:

<i>Eco</i> R1 (hexacutter)	
Adaptor	5'-CTCGTAGACTGCGTACC-3'
	3'-CTGACGCATGGTTAA-5'
Primer	5'-GACTGCGTACCAATTCC-3'
<i>Taq</i> 1 (tetracutter)	
Adaptor	5'-GACGATGAGTCCTGAC-3'
	3'-TACTCAGGACTGGC-5'
Primer	5'-CGATGAGTCCTGACCGAC-3'

¹ Inn-Shik Myung, Overseas Pests Division, Department of Crop protection, National Institute of Agriculture Science and Technology, RDA, Suwon 441-707, Korea.

Fig. 1 Clustering of the strains of *Xanthomonas axonopodis* inducing citrus canker and spot diseases based on their DNA fingerprinting pattern determined by AFLP

DNA template preparation using restriction enzymes *Eco*R1 and *Taq*I and adaptor ligation were done as reported by De Vos et al. [2]. In general 1 µg of DNA was digested with 15 U *Taq*I and 14 U of *Eco*R1 in a final volume of 30 µl. *Eco*R1 and *Taq*I adaptors were added to a final concentration of 0.05 and 0.5 µM respectively and ligated to the restriction fragments in the presence of 0.16 U T-4 ligase and 0.26 mM ATP per reaction tube and they were incubated for 3 h at 37°C. DNA was precipitated with 70% (V/V) ethanol and finally dissolved in 100 µl TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). DNA templates were stored at –20°C. Primers were labelled with [p^{32}] ATP according to De Vos et al. [2]. Performance of PCR reactions, electrophoresis of PCR products, autoradiography, data acquisition and numerical analyses of banding patterns were done as described by Janssen et al. [10].

Results and Discussion

Result of host range determination of the tested strains are shown in Table 2. Based on the pathogenicity tests isolated citrus pathogenic *X. axonopodis* strains from southern Iran were two groups. First group together with Korean strains were pathogenic on all tested plants but strains from the second group showed narrow host range, mainly pathogenic on limes and lemons.

Clustering of the strains based on their DNA binding pattern is shown in Fig. 1, the abridged dendrogram is presented in Fig. 2.

Based on the results of DNA fingerprinting by AFLP tested strains formed six clusters (Figs. 1, 2). The first cluster contained five strains of pathotype C. The second cluster included strains of pathotypes B and D which were closely related to those of the first cluster. Strains of pathotype A and the main group of Iranian strains formed the third cluster. The fourth cluster included strains from South Korea. Strains of pathotype E made the fifth cluster and seven representative strains isolated from affected citrus trees in South of Iran made a completely separate cluster. These strains could be of a new pathotype due to their dissimilarity with those of other groups and their different host ranges. Strains of pathotype B and single available D strain (Mexico) could not be differentiated by DNA fingerprinting [3, 4, 7, 8]. It would be very worthwhile to study more strains from pathotypes D and B to further investigate this close genomic relationship.

AFLP has proven to be useful for identifying bacterial strains at subgeneric level [5, 10, 11, 14, 15, 17, 18, 20]. Although the results of AFLP fingerprinting are specific and show high resolution to differentiate bacterial strains at

Fig. 2 Abridged dendrogram of the strains of *Xanthomonas axonopodis* inducing citrus canker and spot diseases based on their DNA fingerprinting by AFLP

the subgeneric level, it is very clear that the efficacy of this method not only depends on the choice of a suitable restriction enzyme that cleaves the most stable site in the bacterial genome but also depends on application of suitable primer to amplify the unique fragment of bacterial genome which can differentiate one strain from the others. Based on the standard definition the term of pathovar refers to pathogenicity characteristic of the plant pathogen. Pathotypes A, B, C, D and E of *X. axonopodis* could be differentiated through their host ranges and pathogenicity. In our study these pathotypes could be differentiated but forms B, C and D are very closely related to each other and well separated from forms A and E. These results support the previous results published by Vauterin et al. [23, 24]. These pathotypes have at least one or more unique sites on their genomes which can differentiate them from each other. In this study situation is very clear because, except

for strains of pathotypes B and D, all strains could be differentiated based on differences in pathogenicity and DNA fingerprinting.

Iranian strains had different host ranges and they also had different AFLP fingerprinting patterns. The first group of Iranian strains (main group clustered with pathotype A in this study) had a broad host range and a pathogenicity pattern like those of pathotype A. The second cluster (seven representative strains) had narrow host ranges and mainly were pathogenic on Mexican lime (*C. aurantifolia*) and sweet lemon (*C. limettioides*). These seven strains were distinguished by their host ranges and AFLP patterns. Phenotypic features and cellular fatty acids of these seven representative strains were similar to other strains isolated in southern Iran [12, 13]. Both groups were found from all surveyed area in southern Iran. Studied strains from South Korea made a separate cluster (cluster IV) closely related to those of pathotype A and the main group of Iranian strains. These result showed minor differences among the strains from the same pathotype isolated from different geographical areas suggesting genetic diversity among the strains belong to the same pathotype.

References

1. Alizadeh A, Rahimian H (1989) Citrus canker in Kerman province. Iran J Plant Pathol 26:118
2. De Vos P, Hogers R, Bleeker M, Reijans M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414
3. Gabriel DW, Hunter JE, Kingsley MT, Miller J, Lazo G (1988) Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol Plant Microbe Interact 1:59–65
4. Gabriel DW, Kingsley MT, Hunter JE, Gotwald T (1989) Reinstatement of *Xanthomonas citri* (ex Hasse) and *xanthomonas phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. *citri* strains. Int J Syst Bacteriol 39:14–22
5. Gonzalez C, Restrepo S, Tohme J, Verdier V (2002) Characterization of pathogenic and nonpathogenic strains of *Xanthomonas axonopodis* pv. *manihotis* by PCR-based DNA fingerprinting techniques. FEMS Microbiol Lett 215:23–31
6. Gotwald TR, Graham JH, Civerolo EL, Barrett HC, Hearn CJ (1993) Differential host range reaction of citrus bacterial spot determined by leaf mesophil susceptibility. Plant Dis 77:1004–1009
7. Graham JH, Hartung JS, Chase AR (1990) Pathological, restriction fragment length polymorphism and fatty acid profile relationship between *Xanthomonas campestris* from citrus and noncitrus hosts. Phytopathology 80:826–836
8. Hartung JS, Civerolo EL (1987) Genomic fingerprinting of *Xanthomonas campestris* pv. *citri* strains from Asia, South America and Florida. Phytopathology 80:826–836
9. Hartung JS, Civerolo EL (1989) Restriction fragment length polymorphism distinguish *Xanthomonas* strains isolated from Florida citrus nurseries from *X. c. pv. citri*. Phytopathology 79:793–799
10. Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M, Kersters K (1996) Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. J Gen Microbiol 142:1881–1893
11. Janssen P, Maquelin K, Coopman R, Tjernberg I, Bouvet P, Kersters K, Dijkshoorn L (1997) Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting. Int J Syst Bacteriol 46:1179–1187
12. Khodakaramian G, Swings J, Van Eygen S, Stewaert C, Rahimian H (2000) Grouping of the strains of *Xanthomonas axonopodis* inducing citrus canker and spot diseases from Asia, America and Australia on the bases of protein electrophoretic pattern (SDS-PAGE) and phenotypic features (BIOLOG). Iran J Plant Pathol 36:165–178
13. Khodakaramian G, Swings J, Vancaneyt M, Van Eygen S (2000) Fatty acid profiles of the strains of bacteria inducing citrus canker and spot diseases by gas liquid chromatography. Iran J Plant Pathol 36:197–206
14. Kolliker R, Kraehenbuehl R, Boller B, Widmer F (2006) Genetic diversity and pathogenicity of the grass pathogen *Xanthomonas translucens* pv. *graminis*. Syst Appl Microbiol 29:109–119
15. Nesme X, Vaneechoutte M, Orso S, Hoste B, Swings J (1995) Diversity and genetic relatedness within genera *Xanthomonas* and *Stenotrophomonas* using restriction endonuclease site differences of PCR-amplified 16s rRNA gene. Syst Appl Microbiol 18:127–135
16. Pitcher DG, Saunders NA, Owen R (1989) Rapid extraction of bacterial genomic DNA with guanidine thiocyanate. Lett Appl Microbiol 8:151–156
17. Rademaker JL, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P, de Bruijn FJ (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. Int J Syst Evol Microbiol 2:665–677
18. Restrepo S, Velez CM, Duque MC, Verdier V (2004) Genetic structure and population dynamics of *Xanthomonas axonopodis* pv. *manihotis* in Colombia from 1995 to 1999. Appl Environ Microbiol 70:255–261
19. Schoulties CL, Civerolo EL, Miller JW, Stall RE, Krass C, Poe SR, Ducharme EP (1987) Citrus canker in Florida. Plant Dis 71:388–395
20. Simoes TH, Gonçalves ER, Rosato YB, Mehta A (2007) Differentiation of *Xanthomonas* species by PCR-RFLP of *rpfB* and *atpD* genes. FEMS Microbiol Lett 271:33–39
21. Stall RE, Civerolo EL (1991) Research relating to the recent outbreak of citrus canker in Florida. Phytopathology 29:399–400
22. Vauterin L, Swings J, Kersters K (1991) Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. J Gen Microbiol 137:1677–1687
23. Vauterin L, Yang P, Hoste B, Vancanneyt M, Civerolo EL, Swings J, Kersters K (1991) Differentiation of *Xanthomonas campestris* pv. *citri* strains by sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins, fatty acid analysis and DNA–DNA hybridization. Int J Syst Bacteriol 41:535–542
24. Vauterin L, Hoste B, Kersters K, Swings J (1995) Reclassification of *Xanthomonas*. Int J Syst Bacteriol 46:472–489