

Full Length Research Paper

## Characterization of *Xanthomonas citri* subsp. *malvacearum* strains in Iran

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*Xanthomonas citri* subsp. *malvacearum* (*Xcm*), the bacterial blight of cotton is the most important bacterial disease on cotton which infects all aerial parts of the host. Loss due to this disease was estimated for about 10 to 30% on Iranian native cultivars. During summer season, cotton leaves samples with typical bacterial blight symptoms were collected from cotton fields of Golestan province in 2004. Among different bacterial isolates, five distinct *Xcm* isolates were collected for further studies. Besides, 20 partially characterized *Xcm* strains were obtained from Iranian Plant Protection Organization for comparison purpose. Totally 25 strains were subcultured for further characterization based on morphological, biochemical, pathogenicity traits as well as genetic analyses. Among these, nine strains with distinct phenotypic characteristics were selected for complementary genetic studies. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) protein analysis did not show any differences among strains in number and size of protein bands. In plasmid analysis, using alkaline lysis method, a single plasmid was detected for all examined isolates with molecular weight of 23 kb. Repetitive sequence based PCR (rep-PCR) fingerprinting assay using primer BOX AIR (5' CTACGGCAAGGCGACGCTGAC 3') also failed to differentiate the Iranian isolates. It was therefore concluded the strains might have the same origin.

**Key words:** Cotton bacterial blight, protein analysis, plasmid and repetitive sequence based PCR (rep-PCR).

### INTRODUCTION

*Xanthomonas citri* subsp. *malvacearum* (*Xcm*), the causal agent of bacterial blight of cotton (*Gossypium* spp.) is one of the most devastating bacterial diseases which starts as angular leaf spot followed by black canker on stems and boll rot that consequences in discolored lint and infested seeds which are the main source of pathogens transmission (Innes, 1983). Report of this disease in Iran goes back to the years 1959 to 1972 in central and Southern parts of the country (Amani, 1972). After about four decades this disease was reemerged in cotton fields of two main cotton growing provinces of Golestan and Mazandaran (Arab salmani, 2002; Razaghi

et al., 2006). The genome diversity of bacterial plant pathogens including *Xanthomonas* is widely studied with PCR-based methods to point detection, genotypic characterization, ecological distribution and evolutionary process. Thus by analyzing these data, the control and management of the disease would be improved (Goncalves et al., 2000; Robertson et al., 2001).

Among different genetic determinants, the plasmids are one of major genetic elements which have substantial role in virulence of bacteria (Lazo and Gabriel, 1987; Vivian et al., 2001; Sundin, 2007). The full plasmid profile for *Xcm* has been determined with their possible pathogenic role (Sathyanarayana and Verma, 1993). The high degree of preservation was observed among DNA plasmid sequence in some pathovars (Lazo and Gabriel, 1984).

Nara et al. (2004) screened three races of *Xcm* and their

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plasmids size and number were detected respectively for the race 23 (8, 23, 40 and 60 kb), race 27 (4, 8, 23, 40 and 60 kb) and race 32 (2, 4, 8, 23, 40 and 60 kb). The number of plasmids were varied in different growth conditions and when laboratory isolates with fewer plasmids (that is two plasmids of size 60 and 40 kb) were grown in the presence of leaf extract and intercellular fluid obtained from cotton differentials, the number of plasmids were increased to the natural level (Nara et al., 2011). rep-PCR- based approach is also another tool to evaluate genetic diversity of bacterial plant pathogens. This method is valid, reproducible and quick which made it a valuable tool for bacterial identification and classification (Versalovic et al., 1994), specially in respect of genus *Xanthomonas*, rep-PCR is a well discriminatory screening technique to determine genetic diversity of bacterial populations (Rademarker et al., 2000).

In this study phenotypic characteristics, protein analysis by gel electrophoresis and genomic heterogeneity of the isolates were evaluated to reflect a partial integrated approach.

## MATERIALS AND METHODS

### Sampling and isolation

Infected leaves and stems with angular spots were collected from Sahel and Acala cultivars in Golestan province during summer 2004. All sample suspensions were grown on NA and YDC media (Fegan and Hayward, 1983). In total, 25 strains including five screened strains (numbers 1 to 5) plus twenty collected strains (numbers 6 to 25) from Plant Protection Organization of Iran were encoded for further studies.

### Hypersensitivity test on tobacco

This test was done by method recommended by Lozano and Sequeira (1973). Bacterial suspensions ( $10^6$  cfu/ml) were injected into abaxial leaf surface of tobacco leaves.

### Pathogenicity test

The scratched leaves of two local cotton cultivars which are Varamin and Sahel were infiltrated with a suspension of selected bacterial isolates at concentration of  $10^7$  cfu/ml and covered with polyethylene bags for 24 h. The seedlings were further maintained in room temperature and under high moisture conditions for two weeks until the typical symptoms were observed (Innes, 1983).

### Phenotypical and chemotaxonomical tests

The key tests were used to determine phenotypic characteristics of the selected bacterial strains (Schaad et al., 2001). These are including Gram reaction, O/F test, levan and indole production, oxidase, catalase, H<sub>2</sub>S production from cysteine, esculin, starch, gelatin, citrate, Tween 80, casein, arbutin and urea hydrolyses, MR, nitrate reduction, licithinase, reducing substances from sucrose and milk litmus reaction, tolerance of 6% NaCl and 0.1% TTC, acid from arabinose, fructose, galactose, glucose, mannose, sucrose, sodium lactate, acetate and citrate as some of the tested carbon sources and potato soft rot. The viscosity test based on xanthan gums was

also tested (Pierce et al., 1990).  $\geq 26$  s for 0.9 ml to flow through a 1 ml Pyrex pipet was confirmed further characteristics of xanthomonads.

Xanthomonadin production was performed for all strains by solving the mass colonies of the strains in methanol. The supernatants were then spectrophoretically analyzed and absorption spectrum is characterized by maxima at 452 and 480 nm (Starr et al., 1977).

## Determination of genetic diversity

### Total protein analysis

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins of distinct *Xcm* strains were determined in a denaturing discontinuous electrophoresis system which involves a 12% polyacrylamide separation gel and a 5% polyacrylamide concentration gel (Maloy, 1990). The protein profile is visualized by staining with coomassie brilliant blue.

### Plasmid extraction and electrophoresis

To determine the size and diversity of the plasmids, the alkaline Lysis method was used (Morelle, 1989). The bacterial cell pellets were resuspended in 200  $\mu$ l of GTE buffer (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA). 400  $\mu$ l of freshly prepared alkaline solution (0.2 M NaOH, 1% SDS) were added and mixed. After 5 min, 300  $\mu$ l of 7.5 M ammonium acetate solution was added. After centrifugation at 10,000 rpm, 400  $\mu$ l isopropanol was added to supernatants, mixed, and incubate 10 min on ice. Further centrifugation at 21,000 rpm for 15 min led to pellets in turn, were dissolved in 20  $\mu$ l of TE (10 mM Tris pH 8, 1 mM EDTA). 10  $\mu$ l of the aqueous fraction was subjected to electrophoresis on 0.7% agarose minigel with TAE buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM Na<sub>2</sub>-EDTA) at 100 volts for 30 min. The gel was stained with silver nitrate (0.5  $\mu$ g/ml) and the plasmid DNA bands were visualized with a UV transilluminator.

### DNA extraction and running rep-PCR

The DNAs from *Xcm* strains were amplified with single primer of BOX AIR (5' CTACGGCAAGGCGACGCTGAC 3') according to Seal et al. (1993). Box electrophoresis of polymerase chain reaction (PCR) fragments were separated by electrophoresis on agarose gel (1.5%), stained with ethidium bromide (0.5  $\mu$ g/ml) and photographed under ultraviolet (UV) light. PCR standard marker deoxyribonucleic acid (DNA) phage lambda was used with bands at defined intervals of 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

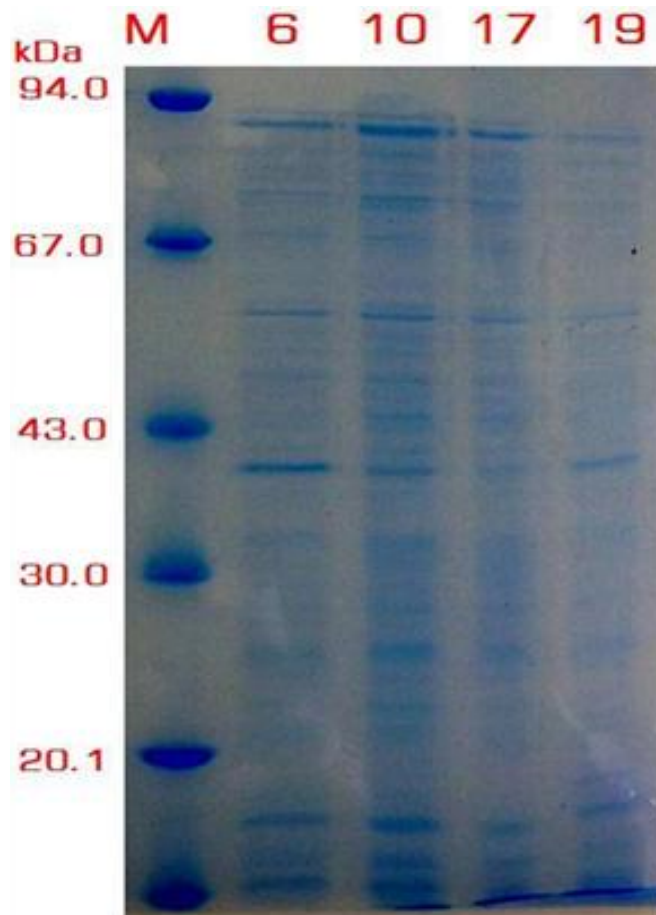
## RESULTS

### Isolation of bacterial isolates

All yellow-colored colonies were isolated from cultured media and individual colonies were purified and stored on yeast dextrose chalk (YDC) medium. Out of fifty isolates, five isolates with distinct colony morphology were selected for collection.

### Hypersensitivity test on tobacco

HR was fulfilled for all *Xcm* strains.



**Figure 1.** SDS-PAGE of total proteins of four *Xcm* strains. Lane A, marker denotes mol. wts. Lane 2-4, local *Xcm* strains encoded 6, 10, 17 and 19.

### Pathogenicity test

All selected strains were induced typical angular leaf spot on treated leaves of Varamin and Sahel cotton cultivars after two weeks.

### Phenotypical and chemotaxonomical tests

No distinct morphological differences in size, shape and color of the colonies were observed. Slight differences were observed in biochemical traits of the strains. All were aerobic with positive reactions to arbutin, indole, licithinase, litmus milk, levan, esculin, starch, cysteine, H<sub>2</sub>S. Casein and lipid hydrolyses, MR reaction, urease and tolerance of 6% NaCl and 0.1% TTC and potato soft rot were negative for all strains. Gelatin hydrolysis test was positive for all but four strains. Arabinose, fructose, galactose, glucose, sucrose, lactate, acetate and citrate were utilized. Trehalose and maltose were negative for all and in five strains, respectively. Xanthomonadin

pigmentation and viscosity test were confirmed for all strains.

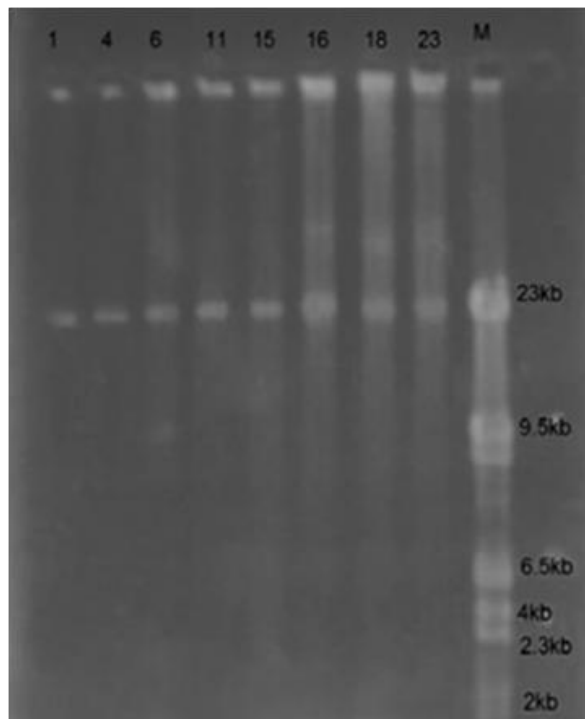
### Determination of genetic diversity

#### *Protein profiles*

Bacterial isolates which showed slight differences in phenotypic characteristics were selected and their protein profiles were compared with the dominant strain. SDS-PAGE of whole-cell protein profiles was completely similar. This revealed that there are no phenotypic differences among the four strains which previously supposed to be different (Figure 1).

#### *Plasmid profiles*

All tested isolates had a single plasmid with a same size. 23 kb plasmid was detected on agarose minigel using



**Figure 2.** Plasmid pattern of selected isolates from bacterial blight of cotton. M, marker. Lanes 1 to 12 *Xcm* strains encoded 1, 4, 6, 11, 15, 16, 18 and 23.

Lambda phage DNA Hind III digest marker (Figure 2).

### Rep-PCR

Genetic fingerprinting by rep-PCR with BOX primers was performed on nine isolates. The genomic rep-PCR profiles consisted of bands ranging in size from 100 bp to 1 kbp (Figure 3). The patterns obtained by rep-PCR analysis were identical for all tested strains.

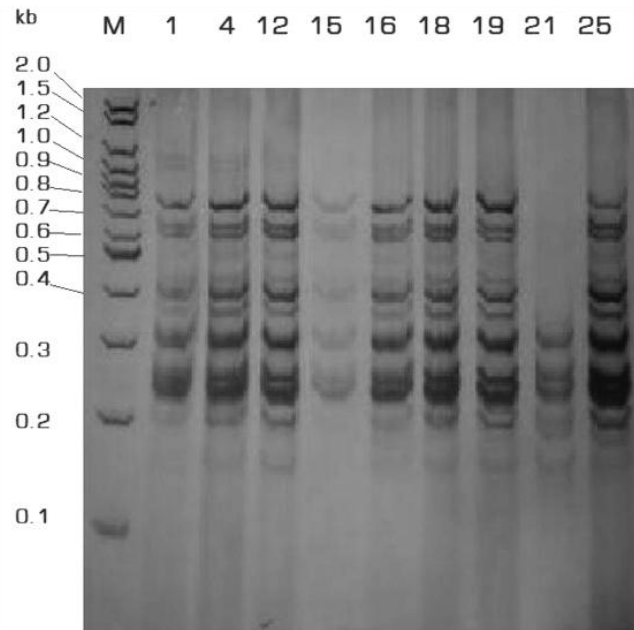
### DISCUSSION

Based on integrated use of phenotypic and genotypic characteristics, the examined strains were identified as *Xanthomonas citri* subsp. *malvacearum*. Although SDS-protein patterns provides a helpful tool to differentiate *Xanthomonas campestris* pathovars (Vauterin et al., 1991), but in our study, biochemical and SDS-PAGE of whole-cell protein profiles could not differentiate the *Xcm* strains.

Plasmid analysis on the other hand, amplified a unique 23 kb fragment of plasmid which was a common character of all isolates. No additional plasmids were detected among Iranian *Xcm* strains. This might be due to typical growth requirements of *Xcm* strains (Narra et

al., 2011). According to Yang et al. (1996), the cotton (*Gossypium* spp.) pathogen *X. campestris* pv. *malvacearum*, has a number of plasmid-borne *avr* genes together redundantly encoded the ability to cause watersoaking in the host plant. Narra et al. (2004 and 2011), reported presence of the same fragment in addition to 4 to 6 plasmids in Indian strains. Irrespective of the number of plasmids, this reveals the importance of plasmid in pathogenicity/virulence of *Xcm*. At present there are a limited number of reports indicating the importance of races, cultivars and nutritional requirements in trends of gaining and losing of the DNA plasmids in *Xcm*. The overall view is a prolonged culturing and repeated subculturing of the bacterial isolates may lead to significant loss of virulence or change in phenotype. This was confirmed by Narra et al. (2011) that showed laboratory isolates which contain fewer plasmids rather than wild strains due to the loss or low number copy during subculturing.

Analysis of enterobacterial repetitive intergenic consensus (ERIC) and rep-PCR among 61 Brazilian isolates of *Xcm* revealed very narrow genetic variability among them with two groups based on the geographic region of isolation (Nunes et al. 2009). In order to assess the global genetic diversity of *Xcm* including highly virulent strains (HVS), 38 strains from different countries were analyzed by rep-PCR with ERIC and BOX primer



**Figure 3.** Electrophoresis of PCR products on agarose gel 0.7% amplified with BOX primer and stained with silver nitrate.

sets which revealed plentiful polymorphism of fingerprinting. The strains with low or moderate virulence were clustered in one genogroup, while all HVSS isolated in Africa were set apart in another genogroup. These results imply that the virulence of *Xcm* highly depends on genotype and or geoclimatic origin of the strains (Zhai et al., 2010).

We found BOX-PCR results unsuccessful in discriminating Iranian isolates. This is because the sole application of BOX primer can only amplify some parts of repetitive extragenic palindromic sequences rather than corresponding to all repetitive DNA sequences.

Perhaps the application of a combined REP/BOX/ERIC PCR could be differentiated the *Xcm* strain at infra-subspecies epithet. Otherwise new developed molecular techniques such as ISSR PCR-based techniques have proven to be suitable and sensitive methods for determination of genetic relationship among *Xcm* strains (Abdo-Hasan et al., 2008).

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## REFERENCES

Abdo-Hasan M, Khalil H, Debis B, MirAli N (2008). Molecular

- characterization of Syrian races of *Xanthomonas axonopodis* pv. *malvacearum*. J. Plant Pathol., 90(3): 431-439.
- Amani B (1972). Black stem disease of cotton. Iranian J. Plant Dis., 8: 112-121.
- Arab salmani M, Rahimian H, Azad F, Ghasemi A (2002). Cotton bacterial blight. Fifteenth Congress of Iranian Plant Protection. Razi University Publisher, Kermanshah, Iran, pp. 119-122.
- Fegan M, Hayward C (1983). Genetic diversity of bacterial plant pathogens. Garland Science/BIOS Science Publisher, Abingdon, UK, pp. 181-204.
- Goncalves ER, Rosato YB (2000). Genotypic characterization of xanthomonad strains isolated from passion fruit plants (*Passiflora* spp.) and their relatedness to different *Xanthomonas* species. Int. J. Syst. Evol. Microbiol., 50: 811-821.
- Innes NL (1983). Bacterial Blight of Cotton. Bio. Rev., 58: 157-176.
- Lazo GR, Gabriel DW (1984). Described races of *Xanthomonas campestris* pv. *Malvacearum*. J. Phytopathol., 74: 837.
- Lazo GR, Gabriel DW (1987). Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. J. Phytopathol., 77: 448-453.
- Lozano JC, Sequeira L (1973). Bacterial blight of cassava in Colombia: Epidemiology and control. J. Phytopathol., 64: 83-88.
- Maloy SR (1990). Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Boston, MA.
- Morelle G (1989). A plasmid extraction procedure on a miniprep scale. Focus, 11(1): 7-8.
- Narra HP, Jayaraman J, Verma JP, Fox RTV (2004). Stability of plasmid profile of highly virulent races of *Xanthomonas campestris* pv. *malvacearum* during storage and subculturing. J. Plant Pathol., 87(2): 171-175.
- Narra HP, Saripalli CS, Gopalakrishnan J (2011). Host induced changes in plasmid profile of *Xanthomonas axonopodis* pv. *malvacearum* races. Afr. J. Biotechnol., 10(3): 2451-2454.
- Nunes MP, Mehta A, Aguiar PH, Cia E, Pizzinato MA, Chiavegato EJ, Mehta YR (2009). Análise da diversidade genética de isolados de *Xanthomonas axonopodis* pv. *malvacearum* do algodoeiro. Summa phytopathol., 35: 105-109.
- Pierce L, Schroth MN, McCain AH (1990). Viscosity test for preliminary identification of strains of *Xanthomonas campestris*. Plant Dis.,

- 74: 646-647.
- Rademaker JLW, 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.*, 50: 665-677.
- Razaghi A, Hasanzadeh N, Ghasemi A (2006). Studies on phenotypic characteristics and genetic variation of *Xanthomonas axonopodis* subsp. *malvacearum* the causal agent of cotton bacterial blight. M.Sc. dissertation, OFIS, Tehran, Iran.
- Robertson AE, Fortnum BA, Wood TC, Kluefel DA (2001). Diversity of *Ralstonia solanacearum* in the Southeastern United States. *Beitrage zur Tabakforschung Int.*, 19(7): 323-331.
- Sathyanarayana N, Verma JP (1993). Possible role of plasmids in the virulence of *Xanthomonas campestris* pv. *malvacearum*. *J. Indian Phytopathol.*, 46: 165-166.
- Schaad NW, Jones JB, Chun W (2001). *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd Ed. APS Press, St. Paul, USA, pp. 17-35.
- Seal SE, Jackson LA, Young JPW, Daniels MJ (1993). Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and the blood disease bacterium by partial 16S rRNA sequencing: Construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. *J. Gen. Microbiol.*, 139: 1587-1594.
- Starr MP, Jenkins CL, Bussey LB, Andrewes AG (1977). Chemotaxonomic significance of the xanthomonadins, novel brominated aryl-polyene pigments produced by bacteria of the genus *Xanthomonas*. *Arch. Microbiol.*, 113: 1-9.
- Sundin GW (2007). Genomic insights into the contribution of phytopathogenic bacterial plasmids to the evolutionary history of their hosts. *Annu. Rev. Phytopathol.*, 45: 129-151.
- Vauterin L, Swings J, Kersters K (1991). Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. *J. Gen. Microbiol.*, 137: 1677-1687.
- Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994). Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Meth. Mol. Cell Biol.*, 5: 25-40.
- Vivian A, Murillo J, Jackson RW (2001). The roles of plasmids in phytopathogenic bacteria: Mobile arsenals? *J. Microbiol.*, 147: 763-780.
- Yang Y, Yuan Q, Gabriel DW (1996). Watersoaking function(s) of XcmH1005 are redundantly encoded by members of the *Xanthomonas avr/pth* gene family. *Mol. Plant-Microbe Interact.*, 9: 105-113.
- Zhai J, Luo Y, Zheng D, Huang X (2010). Evaluation of Genetic Diversity of Highly Virulent Strains of *Xanthomonas campestris* pv. *malvacearum* by rep-PCR Fingerprinting. *J. Phytopathol.*, 158: 764-768.