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Genetic Diversity and Phylogenetic Study of *Xanthomonas arboricola* pv. *juglandis* the Causal Agent of Walnut Bacterial Blight Disease

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Abstract

Bacterial blight (*Xanthomonas arboricola* pv.*juglandis*) is one of the main diseases of walnut that reduce the yield in the central, western and northern regions of Iran. This disease was first reported form, Qazvin and Mazandaran, then widely reported from northern, central and western provinces. To identify the cause of the disease in different provinces, infected leaves collected from Alborze, Lorestan and Kurdestan provinces during spring-summer 2011. Based on the conventional methods of bacteriology, some strains purified and Pathogenicity was proved on three-year plant leaves and on detached fruits of walnut. Based on the biochemical and pathogenesity tests, the yellow colored colonies identified as *Xanthomonas arboricola* pv. *juglandis*. According to the results of cluster analysis model of the rep-PCR method, the strains were categorized in three groups with 56% similarity. On the basis of the sequence of RNA polymerase, beta subunit and dendrogram of NJ method, the strains were differentiated in a separate category. Three strains (10, 11 and 12) were collected from Lorestan and Kordestan and completely separated from other *Xanthomonas* in independent group.

Keywords*:* Rep-PCR, RNA polymeras, Walnut**,** *Xanthomonas arboricola* pv. *juglandis*.

Introduction

The walnut tree is the most important and valuable of hardwood group and nut trees in the world. In some developed countries, it is considered as an important oil tree. Because of its fruit and wood, it has a special importance in the industry (Vahdati and Mojtahedi, 2003). One of the most important factors in reducing the quality and quantity of product are pathogens, especially the walnut bacterial blight which results from the bacterium *xanthomonas arboricola* pv.*juglandis* (Pierce, 1901) (Vauterin *et al.*, 1995) and it has been observed in northern and central provinces of Iran and in many countries that walnut grow. This disease has been reported from many countries (Belisario 1997). The bacterial disease of walnut kernels decay was reported for the first time from Australia by Osborn & Samuel (1922). In Iran, for the first time, it was reported in 1948 by Esfandiyari from northern Iran, then Qazvin (Amani, 1977) and Mazandaran (Mahsoul *et al.*, 1990) and in 1998 widely from northern, central and western of Iran (Golmohammadi *et al.*, 2002). Now, this disease is prevalent in various regions of country.

In 1977, Amani reported the decay of walnut kernels resulted from *X.a*.pv.*juglandis* in Takestan, Qazvin province. This disease infects the leaves, flowers, shoots and fruits and causes heavy losses. *Xanthomonas arboricola* pv. *juglandis* (*X.a.j*) is the causal agent of Persian (English) walnut blight, a disease causing severe economic losses to *Juglans regia* in the worldwide. The repetitive polymerase chain reaction (PCR) and DNA sequencing technique were chosen for this study because of its ability to differentiate bacterial taxa at the strain level (Louws *et al.*, 1994).

Materials and Methods

Sampling and Bacterial isolation

Infected fruit and leaves of walnut were collected from Lorestan, Kordestan and Alborz provinces during spring 2011. The samples were washed and macerated in sterile water for 5 min and a loop of each suspension was streaked.

Separate YDCA media and incubated at 25 ^{o}c After 72 h, single colonies were selected from each sample and cultured on nutrient agar medium. Final identification of isolated was proved in Plant Pathology Department in Iranian Research Institute of Plant Protection, Tehran.

Pathogenicity tests

Hypersensitive reaction in *Tobacco* and *Pelargonium* leaves was tested for bacterial isolates with a cell suspension (10^6 cells/ml) from a 48 h bacterial culture. Pathogenicity test re-confirmed on walnut leaf and fruit (Fig.1).

Biochemical assays

Gram reactions were determined according to standard microbiological protocols (Gerhardt *et al.*, 1994). For carbon source utilization tests, basal Ayers mineral salts medium was prepared according to (Schaad *et al.*, 2001). Various carbon sources were tested by acidification/alkalization on liquid Ayers, with bromothymol blue mixed with different carbohydrates: citrate, D-fructose, D-glucose, D-Dulcitol, sucrose, D-mannose,

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Dulcitol, Oxalate, Cellobiose, L-sorbose, Sorbitol, Benzoic acid. Carbon sources were filter sterilized and added at 0.1-0.5% (w/v) final concentration to autoclaved basal medium. The media inoculated by bacterial strains were incubated at 27^0 C for 48-72 h with 180 rpm shaking.

Tests for arginine dihydrolase, catalase, oxidase, urease, and lipase (Tween 80 hydrolysis) activity assays, tyrosinase, casein hydrolase, aesculin utilization, gelatin liquefaction, starch hydrolysis and formation of indole, formation of levan from sucrose, reducing substances from sucrose, hydrogen sulfide production from cystein, and MR-VP tests were performed according to standard microbiological procedures (Schaad *et al.*, 2001). Growth on nutrient agar, production of fluorescent pigment on KB medium, and growth in presence of 3% NaCl & 4% NaCl were tested accordingly.

DNA extraction and PCR amplification

Bacterial cells were pretreated by the alkaline lysis method to release genomic DNA (Rademaker and de Bruijn, 1997). A cell suspension of desired colony was made in 10 µl of dH2O (about 106 cells/ml). After addition of 100 µl of 0.05 M NaOH, the tubes were incubated in 95 water bath for 15 min. The mixture was then centrifuged for 2 min at 14,000 rpm and the supernatant was used as PCR template.

Rep-PCR analysis

The strains that identified as *Xanthomonas arboricola* pv. *juglandis* were further characterized with fingerprinting patterns generated by BOX and ERIC -PCR. For rep- PCR reaction 1 µl of DNA as template, was added to the reaction mixture. Sterile, molecular grade, ultra-pure water was used in reactions. 1X pcr buffer (incl.1.5 mm MgCl2), dNTP (0.2 mm), the BOX A1R primer [BOX A1R: 5`- CTA CGG CAA GGC GAC GCT GAC G-3` (MWGBiotech, Ebersderg, Munich, Germany)] (50 pmol) and Taq DNA polymerase (MBI, Fermentas, St. Leon-rot, Germany; 2U) and for ERIC-PCR ERIC 1R (5`-ATGTAAGCTCCTGGGGA TTCAC-3`) and ERIC 2 (5`-AAGTAAGTGACTGGGGTGAGCG-3`) primers were used. The PCR was carried out with an initial denaturation at 95 for 2 min followed by 35 cycles that included denaturation at 94 for 3 s and 92 for 30 s, annealing at 50 for 1 min and extension at 65 for 8 min; and the final extension was at 65 for 15 min. Five microliters of each amplification mixture was analyzed by agarose (1.2%, w/v) gel electrophoresis in Tris-acetate-EDTA (TAE) buffer containing 0.5 mg of ethidium bromide per ml (Rademaker *et al.*, 2000).

PCR amplification with rpoB

PCR amplification of *rpoB* gene from bacterial genomic DNA was carried out with primers CM81b-F (5'- TGATCAACGCCAAGCC- 3`) and CM32b-R (5'-CGGACCGGCCTGACGTTGCAT-3`) (Brady *et al.*, 2008). Each PCR reaction mixture contained 1 µl of lysed cell suspension along with 1 × *Taq* buffer, 1.5 mM MgCl2, 0.5 µM of each primer, 0.25 mM of each dNTP and 1.5 U of *Taq* DNA polymerase (Bioron). The reaction mixtures were incubated in a thermocycler (BioRad) at 95^0 C for 5 min, 3 cycles of denaturation at 95^0 C for 1 min, annealing at 55^0 C for 135s and elongation at 72 for 75s, followed by 30 cycles of denaturation at 95^0 C for 35s, annealing at 55 for 75s and elongation at at 72^0 C for 75s and a further 7 min of elongation at 72^0 C. After confirmation by agarose gel electrophoresis, amplicons were sequenced from forward and reverse primers (Parsitek, Iran).

Phylogenetic analysis

Forward and reverse sequences were combined to create a consensus sequence. The sequence results were uploaded to NCBI website (www. ncbi. nlm. nih. gov) to perform a BLAST search. Matching sequences of known organisms with highest scores were selected from Gen Bank along with reference sequences. Further inspection of taxonomic affiliations of obtained sequences was enabled by arranging a multiple sequence alignment of selected data and construction of neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987). Using MEGA5 software (Tamura *et al.*, 2007). The evolutionary distances were computed in the same software using Kimura's two-parameter model (Kimura, 1980). The clustering stability of the NJ tree was evaluated by bootstrap analysis of 1000 data sets.

Results

In this study, 14 isolates from the specimens of leaves, stem, and fruits peel recovered from the provinces of Lorestan, Kurdestan, Alborz and Mazandaran have been isolated on nutritious agar medium. The characteristics of the isolates are shown in (Table 1).

Phenotypic characteristics of the isolates

The isolates on nutritious agar medium were convex and yellow. In all isolates, the gram test was negative. All of the isolates were able to cause hypersensitivity reaction on the leaves of *Tobacco* and *Geranium*. Isolates were aerobic and the production of catalase, hydrolysis of gelatin and esculin in all of them was positive. The production of urease, oxidase, arginine dehydrolase, lecitinase, Potato soft rot in all of the isolates was negative. The isolates were able to produce H_2S from sistein and peptone and gas from Glucose, but above 35°C they were variable between the isolates but they were able to hydrolyze starch. The isolates could tolerate salt %3, but they could not grow on salt %4 environment. They could not grow in culture environment containing %1 TTC too. The isolates were able to produce acid from Glucose and there were differences between the strains in order to have the ability to use

sellobiose, maltose, manose, fructose, citrate, dulcitol, adonitole, oxalate, surbose and sorbitol. According to the results of phenotypic tests, all of the studied isolates were recognized as the isolates of *Xanthomonas arboricola* pv. *juglandis*. According to the results obtained from cluster analysis, the pattern obtained from the isolates based on the phenotypic characteristics showed 3 groups with %70 homology (Fig.3). The results showed that isolates belong to *X.a*.pv. *juglandis.*

Table 1. List of *Xanthomonas arboricola* pv. juglandis isolates obtained from walnut trees in Lorestan, Kurdestan, Mazandaran & Alborz provinces

Sampling site	Isolate No
Mazandaran	X1
Mazandaran	X2
Lorestan	X3
Lorestan	X4
Lorestan	X5
Mazandaran	X6
Alborz	X7
Lorestan	X8
Lorestan	X9
Kurdestan	X10
Mazandaran	X11
Lorestan	X12
Alborz	X13
Alborz	X14

Fig.1. The results of pathogenicity test on walnut plant

Analysis of data obtained from ERIC and REP-PCR

After extraction of DNA from different strains, it was used in polymerase chain reaction. The fingerprint of genomic DNA of different isolates was obtained based on two primer set of ERIC and BOX-PCR. To classify the genotypes, the genetic gap or similarity between isolates was determined and analyzed according to presence (1) or absence (0) of band on gel. The statistical analysis were done using NTSYS-pc software, Version 2/02e, by UPGMA (unweighted pair-group method, using arithmetic averages) and according to Jaccard`s coefficient (Rademaker *et al.*, 2000) (Fig.4).

The number of amplified bands with the primer ERIC was between 9 to 17 bands and the approximate size range was between 150 to 2200 base pairs. The number of amplified bands with the primer BOX was between 7 to 11 band and the approximate size range was between 150 to 1100 (Fig.5). base pair. The dendrogram of isolates similarity based on the results of rep- PCR was merged with BOX and ERIC primers.

The phylogenetic tree was done using related primers designed with house-keeping genes. After PCR, the result of fingerprinting with primer RNA polymerase beta subunit (*rpoB*) was in from of 637 base pair band on the agarose gel (Fig.2). Sequencing of the PCR products was done in both sense and anti-sense directions using mentioned primers with automatic method. The resulted sequences initially corrected with DNAStar ver.5 software and then, applying the BLAST (NCBI) program, they became aligned with the other sequences of gene bank (Altschul *et al.*, 1990). After alignment of pioneer and reverse sequences by DNAStar software, the translation of resulted sequence to protein was done using Chromas

software and the dendrogram of the phylogeny tree was drawn by MEGA5 software with the method of bootstrap-neighbor-joining (Saitou *et al.*, 1987) The results of cluster analysis showed that all isolates of Lorestan and one isolate of Kurdestan were placed in separate groups. These isolates were placed in two separate groups according to beta subunit polymerase RNA gene sequencing and dendrogram drawing with neighbor-joining method. Accordingly, the isolates 10, 11 and 12, which had been recovered from Lorestan and Kurdestan provinces, were separated from the other *Xanthomonases* and placed in an independent group.

Discussion

The results in this study showed that genetic diversity exists among strains of *X.a.* pv. *juglandis* from different geographical areas of the Iran. The studies of former researchers also emphasized the great similarity between different isolates of *X.a.j* from phenotypic characteristic point of view. There were some differences in several bands between the isolates related to protein pattern. It seems that the mentioned method has not a good effectiveness in discrimination of different isolates of *X.a.j* cause of walnut blight. Therefore, we need to assess and introduce the recognition methods in order to exact grouping of plant pathogen bacteria according to the approaches based on genetic fingerprint. That PCR based methods need less time and their stability and effectiveness are more and they are very useful to determine the phylogenetic relationships between the microbial isolates located in one group (Rademaker *et al.*, 1997). In this study, after PCR with rep-PCR and ERIC methods, the fingerprints of isolates were obtained. The cluster analysis of data resulted from mentioned methods using UPGMA method and Jaccard similarity coefficient showed that the X.a.j isolates recovered from different regions of Lorestan, Kordestan, Alborz and Mazandaran provinces, have genetic diversity. The dandrogram obtained from the results of ERIC and rep-PCR also showed that some isolates collected from Lorestan, Kordestan and Mazandaran were placed in a group separated from Alborz province's isolates and reference isolates. Lorestan isolates also showed a different genetic diversity such that they were placed in each of three groups. The results of this study showed that the studied *X.a.j* isolates have different genotypic characteristics. Because the rep-PCR method could reflect the diversity of *X.a.j* different isolates, this method, as a useful and quick molecular method, can discriminate the different isolates of this pathovar which have a close genetic relationship with each other. The results obtained from diversity assessment and evaluation can be used in disease management strategy and epidemiologic and ecologic studies (Louws *et al.*, 1994).

The construction of the similarity matrix showed that no strain from one country had the same profile as that of any strain from another area. Moreover, the overall genetic similarity of the strains from one country was always greater than those of strains from other countries (Scortichini *et al.*, 2001). In this study it has been shown that genetic diversity exists among strains of *X.a.* pv. *juglandis* from different geographical areas of the world. This indicates that each area of Persian walnut cultivation has a different *X.a.* pv. *juglandis* population (Scortichini *et al.*, 2001). The genetic diversity found in *X.a.* pv. *juglandis* strains might also be related to the relatively mild aggressiveness of the pathogen. Although this bacterium in some circumstances can cause economic losses approaching 60% (Olson *et al.*, 1997), it very rarely kills trees. It causes damage to leaves, nut, flowers and twigs, but the plant survives. This also might favor the selection of different genotypes of the bacterium co-infecting the same tree.

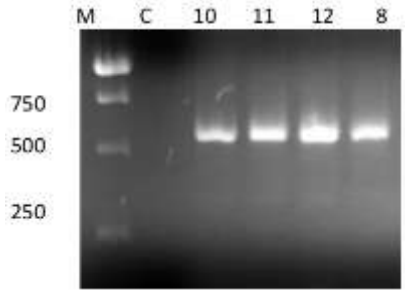

Fig.2. Electerophoretic analysis of PCR product after amplification *rpoB* gen

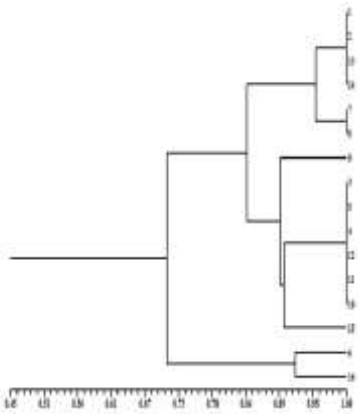

Fig.3. Clustering of 14 *X.a.j* based on similarity coefficients from phenotypic characteristics was generated by UPGMA analysis

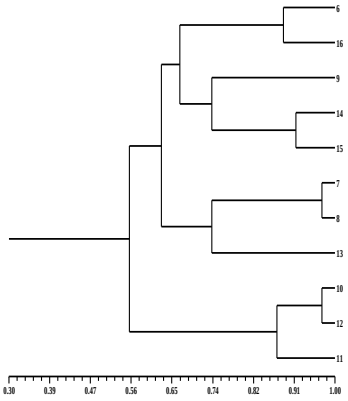

Fig.4. Dendrogram of genetic relatedness of X.a.j The similarity is the result of the combined data set of ERIC and BOX using UPGMA analysis and Jaccard's coefficient.

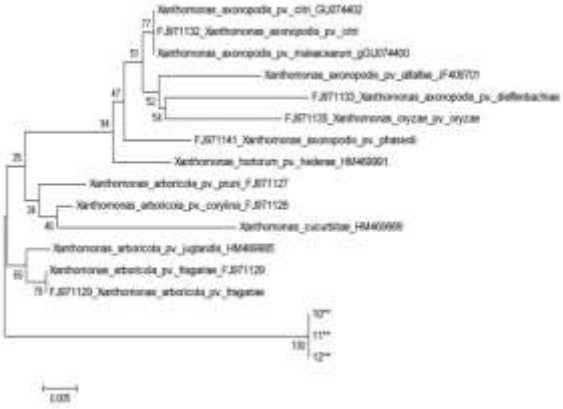

Fig.5. Phylogenetic dendrogram of isolates with Bootstrap –neighbour joining using MEGA5 software

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