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GENETIC DIVERSITY OF *BRENNERIA NIGRIFLUENS* STRAINS IN NORTH OF IRAN (MARGIN OF CASPIAN SEA)

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ABSTRACT. *Brenneria nigrifluens*, the cause of shallow bark canker of Persian walnut trees (*Juglans regia* L.), has become fairly widespread in Iran in recent years. It is regarded as a great threat to walnut production. To determine the diversity of *B. nigrifluens* strains, sixty strains of the causal bacterium were isolated from bark samples of infected walnut trees collected from Mazandaran, Guilan and Golestan provinces and were studied. The physiological and biochemical characteristics, electrophoretic patterns of total cell proteins and rep-PCR generated DNA fingerprints of *B. nigrifluens* strains were compared. Strains appeared to be more or less similar in phenotypic characteristics. Less than 15% of the strains differed in a few phenotypic features such as the ability in production of H_2S from peptone, hydrolysis of esculin, levan production, arginine dehydrolase, nitrate reduction, indol production and methyl red reaction. These differences did not show any special distribution and therefore was not suitable for classifying the strains into distinct groups. The electrophoretic patterns of cell proteins of the strains were different from each other and were only useful for preliminary grouping of the isolates. The groups of strains were differentiated by their rep-PCR fingerprints and on which basis they were placed in six groups in similarity level 95%. Cluster analysis was performed using NTSYSpd software. The results of these studies demonstrated that the populations of *B. nigrifluens* in North of Iran are genetically heterogeneous. The results can be used in selection of disease management strategies.

Key words: Shallow bark canker; Persian walnut; REP-PCR.

INTRODUCTION

One of the important bacterial diseases that affect plants of Persian walnut (*Juglans regia* L.) and can provoke significant reduction in walnut and timber production is shallow bark canker incited by *Brenneria nigrifluens* (Wilson *et al*.,

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1957; Hauben *et al*., 1998). First reported in California (Wilson *et al*., 1957), The disease was also recorded from Spain (Lopez *et al*., 1994), several locations in Italy (Saccardi *et al*., 1998; Morone *et al*., 1998; Scortichini, 1999; Carella *et al*., 2003; Loreti *et al*., 2005) and France (Menard *et al*., 2004). In Iran this disease has been reported from different regions including Mazandaran (Rahimian, 1989; Harighi and Rahimian 1997), Fars and Kohgiluyeh and Boyerahmad (Yousefi Kopaei *et al*., 2004), Kerman (Baradaran and Ghasemi, 2004), Kordestan (Harighi, 2006) and Guilan and Golestan (Jamalzade *et al.,* 2009). This bacterium identified in Korea associated with *Xanthomonas axonopodis* pv. *Pruni* that cause peach & Japanese plum shot hole (Choi *et al*., 2000). In Argentina this pathogen induces Artichoke violet necrosis (Soto, 1997). Also *B. nigrifluens* in Iran isolated from sunflower; but did not prove its pathogenisity (Hassanzadeh and Madjidieh-Ghasemi, 1989).

The disease is mainly characterized by shallow, irregular shaped canker in the bark of the trunk and scaffold branches with dark watery exudates which stained the affected trunk and limb. By removal of phelloderm extensive necrosis of the underlying tissues were observed. In some cases, necrosis extended to cambium and outer sapwood. It can provoke sever damage in young nursery plants (Saccardi *et al*., 1998) and on adult trees (Piccirillo, 2003). Up to now different methods were used for identification of *B. nigrifluens*. For example use of bacteriofages (Zeitoun and Wilson, 1966), study of Serological properties (Harighi and Rahimian, 1997; Yousefi Kopaei *et al*., 2004), study of electrophoretic profiles of total cell proteins (Harighi and Rahimian, 1997; Scortichini, 1999; Yousefi Kopaei *et al*., 2004), Study of fat acids (Wells *et al*., 1994; Scortichini, 1999) and molecular methods for example RFLP (Thoth *et al*., 2001) and Rep-PCR (Moretti *et al*., 2007).

Determination of genetic diversity of a microorganism is important in breeding programs in the search for tolerant or resistant germplasms (Norelli *et al*., 1984). More recently tree families of repetitive sequences including repetitive exteragenic palindromic (REP) sequence, enterobacterial repetitive intergenic consensus (ERIC) sequence and BOX element (Versalovic *et al*., 1994) which are short repetitive DNA sequences have been used to design universal PCR primers that generate highly reproducible, strain-specific fingerprints that can differentiate bacterial strains below the level of species (Little *et al*., 1998). Use of the respective primer (s) in PCR could lead to the selective amplification of distinct genomic regions located between REP, ERIC or BOX sequences.

With attention to increasing trend of this disease in Iran as well as little knowledge about genetic

relationship among isolates of *B. nigrifluens*, the objective of this study was to investigate of genetic diversity of *B. nigrifluens* strains isolated from walnut growing regions in North of Iran (margin of Caspian sea) by using Rep-PCR.

MATERIALS AND METHODS

Sampling and isolation. More than 100 samples of walnut branches and trunk bark with symptoms of shallow bark canker were collected from margin of Caspian Sea (Guilan, Golestan and Mazandaran provinces). For bacterial isolation, the bark surrounding the canker was removed with a flame sterilized knife. Small pieces of tissue, collected with a scalpel at the edge of the cankers, were immersed in a few drops of sterile water contained in plates. After 2-3 h, affected tissues were ground in the plates using steel pestles. A loopfull of the homogenate was streaked onto modified Eosin Methilen Blue Agar (EMB-Agar) containing 1% glycerol and 5% Yeast extract and incubated at 27±1°C. The prevalent bacterial colonies and those similar in appearance to *B. nigrifluens* were selected and purified on NA amended with 5% sucrose. This isolates stored in dionized sterile water and NAS slant tubes at 4°C for next uses. All strains compared with reference strain IBSBF 669T of *B. nigrifluens* in identification tests.

Biochemical, physiological and nutritional tests. All isolates were tested as fallows: Solubility of bacterial cells in 3% KOH (Suslow *et al*., 1982), gram reaction (Schaad *et al*., 2001), oxidative (O) and/ or fermentative (F) glucose metabolism (Hugh and Leifson, 1953), oxidase (Kovacs, 1956), fluorescent pigment (Schaad *et al*., 2001), Hypersensitive reaction in tobacco (Klement *et al*., 1964), Arginine dehydrolase (Thornely, 1960), Methyl-red reaction and starch hydrolysis (Cowan, 1974), Tween 20 hydrolysis (Misaghi and Grogan, 1970), Levan formation, potato soft rot, Gelatin and esculin hydrolysis, catalase, H_2S production from peptone, indol production, reducing substance from sucrose, nitrate reduction, urease production, tolerance to 5% NaCl and growth at 36 and 39°C (Schaad *et al*., 2001).

Protein extraction and SDS-PAGE. For protein extract, bacterial strains grown on NAS for 24 h at 27-28°C were suspended in 1ml sterile distilled water to mid log phase (optical density at 600 nm [OD_{600}], 1).

In order to lyses of cells, 200µl extraction buffer (125mM Tris-HCl [PH 6.8], 4% sodium dodesyl sulfate, 2% mercaptoethanol), added to cells suspension and then they were placed in boiling water for 5 min. After centrifugation at 1300 rpm for 10 min, 40 µl of the supernatant was loaded onto a column. A constant current of 25 mA was used to run gel for 3.5 h. The procedure was performed according to (Laemmli, 1970) using a 5% staking gel (PH 6.8) and a 10% running gel (ph 8.8). Gel were stained for 24 h with 1% coomassie blue R 250, 45% methanol, 45% water and 10% Acetic acid. After 5 h destaining with 10% Acetic acid, gel were immersed in water, photographed, air dried for 24 h and kept (Ausubel *et al*., 1987).

Rep-PCR. Bacterial isolates were subjected to rep-PCR analysis using the primers BOX AIR according to the procedure of (Versalovic *et al*., 1991, 1994) in BIOMETRA termocycler. For DNA preparation, bacterial cells grown on Luria-Bertani broth in shaken culture at 27±1°C according to (Ausubel *et al*., 1987). the amplified products were

electrophoretically separated in 1% agarose gel at 5 V in TBE buffer (10.8 g tris, 5.5 g buric acid, 0.37 g EDTA in 1000 ml dionized sterile water) and visualized with UV light after staining in ethidium bromide (0.5 µg/ml). The molecular sizes of fragments generated were estimated by comparison with simultaneously run Ladder Mix (GeneRuler^{TM} DNA Ladder Mix, Fermentas). Lanes were compared by reading horizontally across the gel image, from bottom to top; if a band was present, it was assigned a value of 1 at that location, whereas if absent, it was assigned a value of 0. The presence/absence of bands was collated into a binary data matrix. Cluster analysis was performed on similarity matrices which were produced using similarity coefficient and subjected to the between group linkage method using NTSYSpc software, version 2.1. To establish robustness of clusters, the cophenetic value was obtained using the NTSYSpc software.

RESULTS AND DISCUSSION

Isolating and phenotypic properties. A total of 104 isolates from branches and trunk bark with symptoms of shallow bark canker were collected from walnut growing regions of margin of Caspian sea and on the basis of biochemical, physiological and nutritional tests, 60 isolates identified as *B. nigrifluens*. Collected regions and number code of these strains showed in *Table 1*. Morphological, biochemical and nutritional properties of this isolates showed in *Table 2*.

Strains produced a green metallic sheen on modified EMB and spherical, white with smooth margin on NA amended 5% sucrose after 2-3 days at 27 ± 1°C. Some isolates in first culture on modified EMB produced purple color, but in subculture produced green metallic sheen again, this isolates had further growing rate in compare to other isolates, but were not different in biochemical, physiological and nutritional properties. A few (less than 15%) isolates were different in some properties such as Levan production, Esculin hydrolysis, H_2S production from peptone, Arginine dehydrolase, Nitrate reduction, Indol production and Methyl red reaction. These differentiations had not specific distribution and clustering of this isolates on this basis was impossible. Other properties of isolates gave the same results of those obtained with the *B. nigrifluens* reference strain (IBSBF 669T). Results of phenotypic properties of isolates were according to identified properties for species of *B. nigrifluens* (Wilson *et al*., 1957; Bradbury, 1981; Lelliott and Dickey, 1984; Verdonck *et al*., 1987). In starch hydrolysis test, Iranian strains even after 10 days had not this ability that this occurring to Harighi and Rahimian (Harighi and Rahimian, 1997) results and opposed to Wilson *et al*. results (Wilson *et al*., 1957). On the basis of biochemical, physiological and morphological properties, this isolates were very similar and had low differentiation in some phenotypic properties. Specific relationship did not exist among phenotypic distinguishing and collecting regions.

Table 1 - Locations and code number of *Brenneria nigrifluens* strains isolated from walnut trees in Mazandaran, Gilan and Golestan provinces

Code no.	Location	Code no.	Location
Bn1*	Eshkar Dasht, Chalus	Bn31	Baye Mahale, Joibar
Bn2*	Badele, Sari	Bn32	Ghaem Shahr
Bn3*	Astane, Rasht	Bn33	Joibar
Bn4*	Amlash	Bn34	Ghaem Shahr
Bn5	Astane, Rasht	Bn35*	Ghaem Shahr
Bn6*	Pasand, Behshahr	Bn36*	Joibar
Bn7	Pasand, Behshahr	Bn37*	Joibar
Bn8	Rostam Kola, Behshahr	Bn38*	Ghaem Shahr
Bn9*	Badele, Sari	Bn39*	Haris, Tonekabon
Bn10	Eshkar Dasht, Chalus	Bn40*	Moalem Kooh, Tonekabon
Bn11*	Shahid Abad, Behshahr	Bn41*	Badele, Sari
Bn12*	Darab Kola, Sari	Bn42	Badele, Sari
Bn13*	Tonekabon Shahidabad,	Bn43	Ali Abad-E- Katool
Bn14*	Darab Kola, Sari	Bn44	Ali Abad-E- Katool
Bn15*	Chalus, Noshahr	Bn45	Kordkoy
Bn16	Baye Mahale, Joibar	Bn46*	Kordkoy
Bn17*	Baye Mahale, Joibar	Bn47	Fazel Abad, Gorgan
Bn18	Chalus, Noshahr	Bn48*	Bandar Gaz
Bn19	Haji Abad, Amlash	Bn49	Ali Abad-E- Katool
Bn20*	Chalus, Noshahr	Bn50*	Bandar Gaz
Bn21*	Haji Abad, Amlash	Bn51	Gelin, Ali Abad-E- Katool
Bn22	Layem, Sari	Bn52	Bandar Gaz
Bn23	Douseyedan, Astane	Bn53	Bandar Gaz
Bn24	Rostam Kola, Behshahr	Bn54	Bandar Gaz
Bn25*	Chaboksar	Bn55*	Badele, Sari
Bn26*	Roodsar	Bn56*	Badele, Sari
Bn27	Ghaem Shahr	Bn57	Badele, Sari
Bn28*	Joibar	Bn58	Ramsar
Bn29*	Zaghmarz, Sari	Bn59	Bahnamir, Babolsar
Bn30	Babol	Bn60	Lahejan

***** Isolates used for investigation of electrophoretic profiles of total cell proteins and BOX-PCR

Table 2 - Morphological, biochemical and physiological characteristics of *Brenneria nigrifluens* strains isolated from walnut trees in Mazandaran, Gilan and Golestan provinces

Reaction	Characteristics	Characteristic of reference isolate
Gram reaction	-	-
Oxidase	-	-
Catalase	+	+
Levan formation	-(96%)	-

Reaction	Characteristics	Characteristic of reference isolate
Fluorescent pigment	-	-
Tolerance to 5% NaCl	+	+
Gelatin hydrolysis	-	-
Esculin hydrolysis	+(91%)	+
Arginine dihydrolase	-(95%)	-
Starch hydrolysis	-	-
Teween 20 hydrolysis	-	-
Growth at 36°c	+	+
Growth at 39°c	-	-
H_2S production from peptone	+(90%)	+
Urease production	+	+
Potato soft rot	-	-
Nitrate reduction	-(85%)	-
Indol production	-(85%)	-
Production of blue pigment on YDC	-	-
Methyl-red reaction	+(93%)	+
Reducing substance from sucrose	-	-
Hypersensitive reaction on tobacco	-	-
Acid from:		
Ribose	+	+
Xylose	+	+
Arabinose	+	+
Ramnose	+	+
Maltose	-	-
Sorbose	-	-
Raffinose	+	+
Cellobiose	+	+
Manitol	-	-
Lactose	-	-
Dulcitole	-	-
Adonitol	-	-
Erythritol	-	-
Sucrose	+	+
Glyserol	+	+
Utilization of:		
Galacturonate	-	-
L-Tartarate	-	-
D-Tartarate	-	-
Citrate	-	-
Formate	-	-
Oxalate	-	-
Ascurbate	+	-
Benzoate	-	-
Acetate	-	-
Ethanol	-	-
Propanol	-	-
Lactate		-
Succinate	+	+
Propionate	-	-

Reaction	Characteristics	Characteristic of reference isolate
DL-Alanine	-	-
L-Leucine	-	-
L-Serine	-	-

% percentage of react strains; + positive reaction; - negative reaction

Protein electrophoretic pattern. Randomly 30 isolates of *B. nigrifluens* (marked isolates in *Table 1*), selected from several locations of walnut- growing regions of North of Iran and compared to each other and to reference strain of *B. nigrifluens* by electrophoretic profile of cell protein *(Fig. 1)*. On which basis isolates had different electrophoretic pattern and were only useful for preliminary grouping of the isolates. Differentiation in protein electrophoretic pattern of isolates of Mazandaran province was reported earlier (Harighi and Rahimian, 1997).

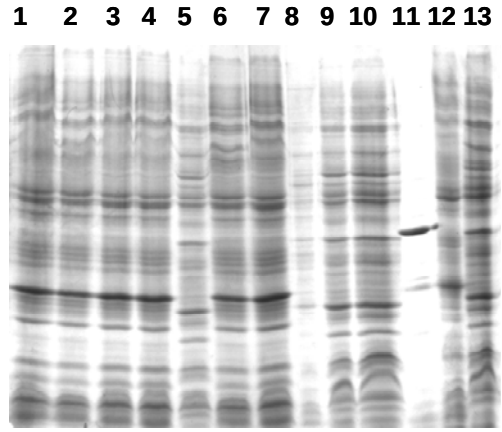

Figure 1 - Electrophoretic profile of the total cell proteins of *Brennerria nigrifluens* strains isolated from walnut trees in Mazandaran, Gilan and Golestan provinces in polyacrylamide gel. Strains were; 1: Bn48; 2: Bn50; 3: Bn11; 4: Bn41; 5: Type strain (IBSBF669); 6: Bn39; 7: Bn14; 8: Bn25; 9:Bn28; 10: Bn17; 11: Bn12; 12: Bn2; and 13: Bn6

BOX-PCR analysis. The DNA fingerprints of 30 isolates (marked isolates in *Table 1*) were determined by using BOXAIR primer in PCR *(Fig 2).* BOX-PCR analysis produced the dendrogeram shown in *Fig. 3*. The genomic BOX-PCR profiles consisted of bands ranging in size from 200 to 2500 bp. Reproducible, clearly resolved bands numbers were 2 to 16 bands. Clustering analysis showed that investigated isolates were placed in six groups in similarity level 95%: Group 1: Bn12, 20, 21; Group 2: Bn40; Group 3: Bn41, 55; Group 4: Bn39; Group 5: Bn35; Group 6: Bn2.

The results demonstrate existence of genetic heterogeneity in the population of *B. nigrifluens* in northern Iran.

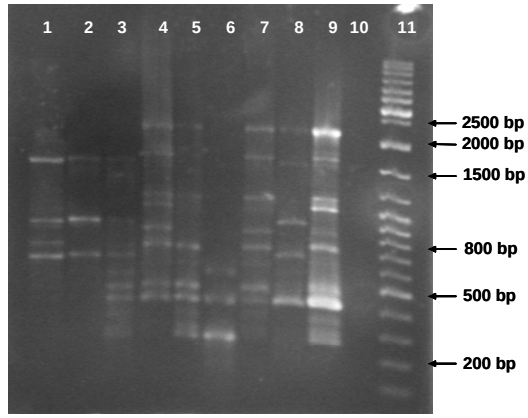

Figure 2 - Electrophoretic patterns of amplified DNA of *Brenneria nigrifluens* strains isolated from walnut trees in Mazandaran, Gilan and Golestan provinces using BOXA1R primers in 1% agarose gel. Strains were 1: Bn39; 2: Bn41; 3: Bn2; 4: Bn40; 5: Bn12; 6: Bn35; 7: Bn20; 8: Bn55; 9: Bn21; 10: negative control; and 11: DNA-Ladder

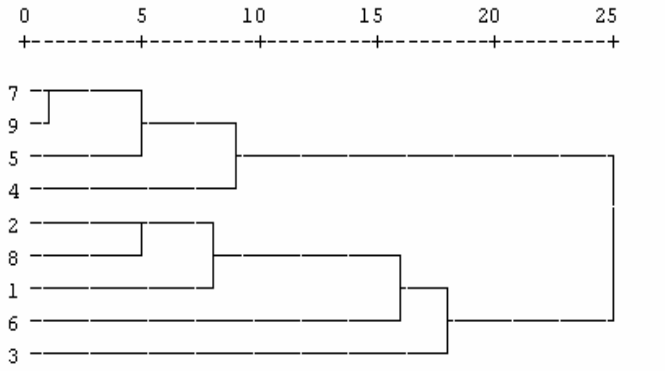

Figure 3 - Dendrogram of *Brenneria nigrifluens* strains isolated from Mazandaran, Gilan and Golestan provinces on the basis of genetic relationship of fingerprints of the BOXA1R primers (1: Bn39; 2: Bn41; 3: Bn2; 4: Bn40; 5: Bn12; 6: Bn35; 7: Bn20; 8: Bn55; 9: Bn21)

Rep-PCR revealed genetic heterogeneity in the population of *B. nigrifluens* in northern Iran. Selection for a specialized niche could affect the distribution of repetitive sequences, appears to fingerprints unique to specific strains present in a given area (Louws *et al.*, 1994). Rep-PCR

technique comparison with other PCR-based genomic fingerprinting such as RAPD analysis is more suitable. Although the RAPD technique is commonly used, problems of reliability and low reproducibility with this marker exist. Length of primers used in RAPD analysis most commonly 10mers but up to 34 bp, whereas Rep-PCR involves the use of primers of 18 to 22 bp with high homology to repetitive sequences that allow the use of more stringent PCR conditions, which in turn may reduce experimental variation and PCR artifact (Louws *et al*., 1994). REP-PCR has been shown to be a quick and reliable technique to differentiate plant pathogenic bacteria at or below the species level with highly reproducible results (Versalovic *et al*., 1994). In this method, the prior knowledge of target DNA sequence is not necessary (Louws *et al*., 1994). This study confirmed that the Rep-PCR technique is a rapid, simple and reproducible method in assessment of the diversity of *B. nigrifluens* strains.

The genetic diversity found in *B. nigrifluens* population might be related to the relatively mild aggressiveness of the pathogen. Moreover with attention to cultivation of different walnut ecotypes in different regions, these ecotypes could have selected different *B. nigrifluens* population. Symptoms of walnut bark canker disease in Iran frequently were observed on adult trees (10-15 years old); in activity (28°C), seems pathogen agent has the highest activity in this temperature domain and upper and lower temperature level cause decrease in bacterial activity. Also affected trees frequently survive; this also may favor the choice of distinct genotypes of the bacterium co-infecting the same tree addition symptoms on trees mainly were observed on oldest parts of tree (trunk of walnut trees). However this disease not only associated to adult trees and in some cases symptoms of disease was observed on young nursery plants. In addition, in preliminary years of distribution of this disease, activity of this bacteria associated to summer season; survey in 2008 showed that disease agent activity to middle Autumn, however with regarded to the optimum growing temperature and bacterial activity (28°C), seems pathogen agent has the highest activity in this temperature domain and upper and lower temperature level cause decrease in bacterial activity. Also affected trees frequently survive; this also may favor the choice of distinct genotypes of the bacterium co-infecting the same tree.

CONCLUSION

Heterogeneity exists among the population of *B. nigrifluens* in North of Iran and Rep-PCR technique is a rapid, reliable and reproducible in assessment of the diversity of *B. nigrifluens* strains.

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