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Identification of deep bark canker agent of walnut and study of its phenotypic, pathogenic, holotypic and genetic diversity in Iran

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ABSTRACT

The deep bark canker of the walnut is a serious threat to walnut plants that had an increasing verge since recent years. This disease is caused by *Brenneria rubrifaciens* bacteria. Therefore, this research was carried out with an aim to identify the deep bark canker agent. The suspected samples having symptoms were collected from the bark and branches of walnut trees. After purification, the phenotypic and biochemical specification of separations was assessed. Even the genetic diversity of pathogen population was conducted with BOX-polymerase chains reaction (PCR) technique and BOXAIR primer. Based on the phenotypic and biochemical specifications, 30 isolates were identified as *B. rubrifaciens*. In PCR reaction, the specific amplicons of size 537 and 671 bp in the case study strains were amplified via specific primer pairs. The present study is a prime report on outbreak of this disease in Iran and bacterial pathogenicity evidence of *B. rubrifaciens* on fruit in the world.

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Brenneria rubrifaciens; walnut canker; genetic diversity; BOX-PCR

Introduction

Iran is a fertile land with variant and diverse ecosystem that includes a part of diversified cultivations of walnut globally. This climate and ecosystem diversity can be less found in other points in the world. The walnut is one of the most invaluable plants that has wood, fruit, industrial, foreign exchange importance besides its other useful specifications and plays an important role in economic of the country. In countries wherein walnut is cultivated on widespread level, different diseases can be observed that introduce irreparable damages to the walnut trees (Jamalzade et al. 2012).

One of the most important diseases of the walnut is that caused by a deep bark canker which in high moisture and temperature leads to reduction of quality and production rate of walnut. Disease symptoms include the presence of craks with a depth of several centimeters along with a brown- to black-colored latex discharge whereby these cankers are primarily superficial and gradually get deeper (McClean et al. 2014). In 1967, the severe and deep cankers were reported on the bark of walnut trees in the California provinces that were denoted as deep cankers of walnut and the causative agent was described as *Brenneria rubrifaciens* (Wilson et al. 1967). The bacterial agent of walnut deep trunk canker was also assessed via Kado and Garden. According to the mentioned investigation, host sensitivity in relation to disease agent is dependent on contamination period, presence of wound and initial inoculum rate (Kado and Garden 1977). In a study, the researchers, using biochemical, pathogenicity and Elisa test, reported *B. rubrifaciens* bacterium for the first time in Spain and Europe, with conduction of pathogenicity tests, showed that the disease agent bacterium after four years of contamination creates wounds with a depth of 8–20 cm in the walnut tree bark (González et al. 2002).

Among the most important differences of the walnut bacterial canker agent with other bacterial canker agents in plants is the presence of pigments *rubrifaciens* in the bacterium *B. rubrifaciens*; this pigment has a role in the pathogenicity of pathogen. Feistner et al. (1982) reported for the first time the separation of *rubrifaciens* from *B. rubrifaciens* of walnut plants and described the extraction mode, the characteristics of this pigment and function mechanism (prevention of transfer to electron in the mitochondria).

Control of diseases due to herbal pathogen bacteria generally needs precise diagnosis followed by correct identification of the disease-causing organism, where methods based on the nucleic acid sufficient sensitivity is used (Stoger et al. 2006). At present, a wide range of herbal pathogenic bacteria have been identified using polymerase chain reaction in numerous hosts (Loreti et al. 2008). In the recent two decades, based on repetitive sequences present in genome of different types of bacterial species, primers have been designed to create genetical fingerprinting effect, which are useful in the assessment of diversity in strain and pathovar level (Tvasoli et al. 2009).

Three groups of repetitive sequences REP (Repetitive extragenic palindromic), BOX and ERIC (enterobacterial repetitive intergenic consensus) were studied in detail, wherein with the designs of some primers based on these repetitive sequences were introduced as REP-PCR, BOX-PCR and ERIC-PCR. The replication of genome-specific areas and the obtained genomic pattern from these methods led to the production and the distinction of amplicons with different sizes of DNA isolate-specific genome, which is used as an identifier for each of the specific isolates (Oliver and Bean 1999). However, sufficient information about the molecular diagnosis techniques in relation to *B. rubrifaciens* bacteria is not accessible.

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During the past few years with an increase in cultivation level of walnut trees in Iran, the symptoms of bacterial diseases of walnut in orchards have increased. Considering the lack of study in correlation with walnut deep trunk canker in Iran and the importance of genetic diversity in epidemiology and disease management, the present research was carried out with an aim to separate and diagnose disease agent of deep trunk canker of walnut and based on the phenotypic and molecular tests using specific primers pair and determination of pathogenicity power and genotypic diversity using BOX-PCR method.

Material and methods

Sampling, separation and maintenance of disease agent

During spring and summer of 2014 and 2015, 140 suspected herbal samples having wound symptoms were collected from the bark and branches of walnut plants from main walnut cultivation regions and were separately transferred to laboratory in plastic bags. For separation of bacteria, the canker and latex on sample of bark and branch was used. For this reason, the pieces in the limit between healthy and contaminated tissue of each sample was prepared and washed several times with sterile distilled water. Later the washed pieces were inserted in the Petri containing sterile water and were shaken for 24 h. Afterwards, a loop of bacterial suspension was cultivated on NA (Nutrient Agar) medium in linear form and incubated at 28°C for 24 h. After the growth, white- or cream-colored round colonies were selected and cultured on EMB (Eosin Methylene Blue) and YDCA (Yeast Extract Dextrose Calcium-Carbonate Agar) (Merck Co., Germany) media and were incubated at 28°C. Among the key and important specifications of these bacteria was the formation of red to pink pigments on YDC medium and production of green metallic pigments on EMB medium. After three days, the colonies on YDC and EMB media that created characteristics of *B. rubrifaciens* bacterial colonies were selected and for purification were recultured on NA medium. For further studies, the purified colonies were maintained in sterile distilled water and 15% glycerol at 70°C.

Investigating the phenotypic and biochemical characteristics of isolates

The collected isolates from walnut trees that had metallic green colonies on EMB medium and were able to form red- to pink-colored pigments on YDC medium and the formation of hypersensitivity on geranium and tobacco as a representative were selected and Gram, oxidase, catalase, urease, methyl red, starch hydrolysis, gelatin hydrolysis tests besides other important tests namely physiological, biochemical and nutritional was conducted on them based on the herbal bacteriology standard methods (Schaad et al. 2001).

The pathogenicity test on walnut sapling and fruit

In order to validate pathogenicity of isolates, inoculation was carried out on two-year-old walnut saplings. To inoculate the bacteria, four isolates were selected and after adjustment of the bacterial suspension concentration to 10^8 cfu/ml (optic density 1.5 in the 600 nanometer wavelength – spectrophotometer model Unico, U.S.A.), using a 60 µl sterile syringe, the bacterial suspension was inoculated into trunk and young branches. The achieved wounds locale was shielded with a Parafilm tape. In control sapling, the sterile distilled water was used as inoculate. The saplings were observed for three months. In order to conduct pathogenicity test, immature walnut fruits prior to crust hardening were collected from 20- to 30-years-old walnut trees at the end of spring and summer of 2014. Five bacterial isolates that were diagnosed in biochemical and molecular tests of *B. rubrifaciens* were selected to conduct pathogenicity test on immature fruit. From the 48-h-old bacterial culture, a suspension with a concentration of 10^8 cfu/ml (optical density 1.5 in the 600 nanometer wavelength – Spectrophotometer model Unico, U.S.A.) to 10^2 cfu/ml (optical density 0.3 in 600 nanometer wavelength – Spectrophotometer model Unico, U.S.A.) was prepared in the sterile distilled water; using a sterile syringe the fruit surface was scratched (three holes in the fruit), and inside each hole 50 µl of suspension was inoculated. For each concentration of bacterial suspension, a control inoculated with sterile distilled water was considered. Later, the fruits were maintained in germinator at 20°C, and relative humidity of 90% and optical duration of 12 h light for eight days (Moretti and Buonaurio 2010).

Polymerase chain reaction

In order to amplify asparagine synthetase gene in expected region 537 bp of primer pairs BrAF (ATGTACGCAGTCTCTATTTGG) and BrAR (CCATCAGCCTGAAATAACTCA) and to amplify autoinducer synthase gene in expected region, 671 bp of primer pairs 2BrIF (CGGGATCCATGTTAGAAATATTCGATGTC) and 2BrIR (TCAGCTGTCAAGCCTCTTCCTTTTTG) was used (Thapa et al. 2010). These genes have a role in the production of pigment.

For conduction of polymerase chain reaction, a loop of bacterial colonies on NA medium was taken and dissolved in 100 µl of sterile distilled water. For DNA extraction, the samples were boiled for 10 min in a container containing boiled water and immediately were placed on ice for a few minutes. Later the samples were centrifuged at 13,000 rpm for 5 min and upper phase was used for PCR test.

Polymerase chain reaction was carried out in 25 µl volume including 2.5 µl of 10 × PCR buffer (500 mM KCl, Tris–HCl, acidity 8.4), 20 pmol of each primer, 0.2 mM dNTPs (Deoxynucleoside triphosphate), 1.5 mM MgCl2, one unit of Taq Polymerase (Sinagene, Iran) and 2 µl DNA pattern in the thermocycler system Bio Red (U.S.A.) model. The preliminary annealing was carried out at 94°C for 2 min. PCR thermal steps in 30 cycles including genomic DNA denaturation at 94°C for 45 s, the attachment of primer to pattern string at 62°C and 64°C for 30 s for primers BrAF/BrAR and 2BrIF/2BrIR, the extension of new strand at 72°C for 1 min and at the end of final elongation at 72°C for 7 min.

The BOX-PCR reaction was assessed using BOXAIR (CTACGGCAAGGCGACGCTGACG) primer (Versalovic et al.1994). The total amount of each reaction was 25 µl, including 2.5 µl of 10 × PCR buffer, 20 pmol of primer, 0.3 mM dNTPs, 1.5 mM of MgCl2, 3 µl pattern DNA and 2 units of Taq DNA polymerase. The thermal cycle with preliminary DNA denaturation at 95°C for 3 min was initiated and 35 cycles included DNA denaturation at 95°C for 30 s,

primer attachment at 53°C for 1 min, extension at 65°C for 8 min and eventually the final elongation at 65°C for 16 min (Versalovic et al. 1991; Versalovic et al. 1994). The electrophoresis of BOX-PCR was carried out in 1.5% agarose gel and in TBE (Tris/Borate/EDTA) (1×) buffer for 2 h and constant voltage of 80 V. After the electrophoresis of BOX-PCR products, the gel was photographed via Gel documentation. The sterile distilled water was used as a negative control.

Data analysis

Using the Numerical Taxonomy and Multivariate Analysis System Ntsys-pc version 2.02e software, the distance or genotypic similarity of isolates was traced. For real investigation among clusters, the Unweighted Pair – Group Method using Arithmetic average method and Jaccard similarity coefficient were adopted (Rohlf 2000). The genotypic characteristics for this software were defined as code one for the presence of bands and code zero for the lack of bands. The similarity percent was calculated based on the entire characteristics present in this research and dendrogram related to 12 representatives among the case investigated was traced via this software.

Results

Separation of disease agent

According to present study results and based on the bacteriology standard tests, in total 30 isolates were identified as *B. rubrifaciens* bacteria from different parts of walnut trees. The amplification regions of *B. rubrifaciens* strains separated in this assessment is tabulated in Table 1.

The physiological, biochemical and nutritional characteristics of isolates

Based on these characteristics tests, *B. rubrifaciens* bacterial strains identified from walnut trees in Iran were assessed. The results of some characteristics included negativity of Gram and oxidase reaction, positivity of catalase, methyl red and facultative anaerobes and ability to produce H2S gas from python and reducing substance from sucrose, ability to use citrate, besides the ability to produce metallic green pigment on EMB medium, red- to pink-colored pigment on YDC medium and pink pigment on King B medium. None of the isolates were able to hydrolyze gelatin and starch. The results of tests in general are tabulated in Table 2.

Table 2. Morphological, biochemical and nutritional characteristics of *B. rubrifaciens* strains separated from walnut plants in Iran.

Test	Reaction	Test	Reaction
Gram	–	Gas production from glucose	–
Oxidase	–	Endospore production	–
Catalase	+	Production of acid from	
Levan	–	Ribose, sucrose, lactose, adonitol and xylose	+
Hypersensitivity on tobacco	+	Arabinose and cellulose	+[b]
Gelatin hydrolysis	–	Raffinose and rhamnose	–[b]
Esculin hydrolysis	+[b]	Use of:	
Swarming movement of bacteria	+	Fumarate	
Indol	–	Benzoate	
Tween 20 hydrolysis	–	Propanol	
Starch hydrolysis	–		
Methyl red reaction	+	Succinate and lactate	+
Production of H2S gas from python	+	Ethanol	–
Growth at 36°C	+	Oxalate	–
Growth at 40°C	–	D-tartrate	–[a]
Urease	–	L-leucine	–
Pink pigment on KB medium	+	Mannitol	+
Red pigment on YDC medium	+	Dolistol	–
Metallic green pigment on EMB	+	L-Valine	+
Potato rot	+	Citrate	+
Reducing substances from sucrose	–	L-serine	–
O/F	+	DL-alanine and DL-tartrate	–
Nitrate reduction	–	Melonate	–

+: indicates positivity of the reaction, –: indicates negativity of the reaction
[a]Over 70%.
[b]Over 90%.

Identification of isolates using specified primers of B. rubrifaciens

To validate present results, the phenotypic results of PCR reaction were used. In the polymerase chain reaction, a genetic template related to asparagine synthetase gene with an extension of 537 bp and genetic template related to autoinducer synthase gene with an extension of 671 bp was amplified in 35 isolates. Besides, the templates size in the case of the entire samples was similar and in this regard no

Table 1. Bacterial strains used in this experiment.

Isolates	Source	Tissue	Date isolated	Isolates	Source	Tissue	Date isolated
RA1[a]	Rasht	Trunk	May 2014	LO1[a]	Borujerd	Trunk	August 2014
RA2	Rasht	Branch	May 2014	LO2	Aleshtar	Trunk	August 2014
RA3	Rasht	Trunk	May 2014	LO3	Dorud	Trunk	August 2014
BE2	Behshahr	Trunk	June 2014	HA1	Hamedan	Trunk	September 2015
BE3[a]	Behshahr	Branch	June 2014	HA2	Hamedan	Trunk	September 2015
SA2	Sari	Branch	May 2015	HA3[a]	Hamedan	Branch	September 2015
SA3[a]	Sari	Trunk	May 2015	ES1	Esfahan	Trunk	July 2014
NO4[a]	Noshahr	Trunk	August 2015	ES2[a]	Esfahan	Trunk	July 2014
GO1	Gorgan	Trunk	June 2014	ES3	Esfahan	Trunk	July 2014
GO2[a]	Gorgan	Branch	June 2014	YA2[a]	Yasooj	Branch	September 2015
CH1[a]	Chaloos	Trunk	July 2015	SH1[a]	Shiraz	Trunk	September 2015
CH2	Chaloos	Branch	July 2015	JA2	Jahrom	Trunk	September 2015
TO1[a]	Tonekabon	Branch	July 2015	KE1	Kerman	Branch	August 2015
TO2	Tonekabon	Trunk	July 2015	BA2	Babol	Trunk	June 2015
TO3	Tonekabon	Branch	July 2015	BS1	Babolsar	Branch	June 2015

[a]Strains used in this BOX-PCR reaction.

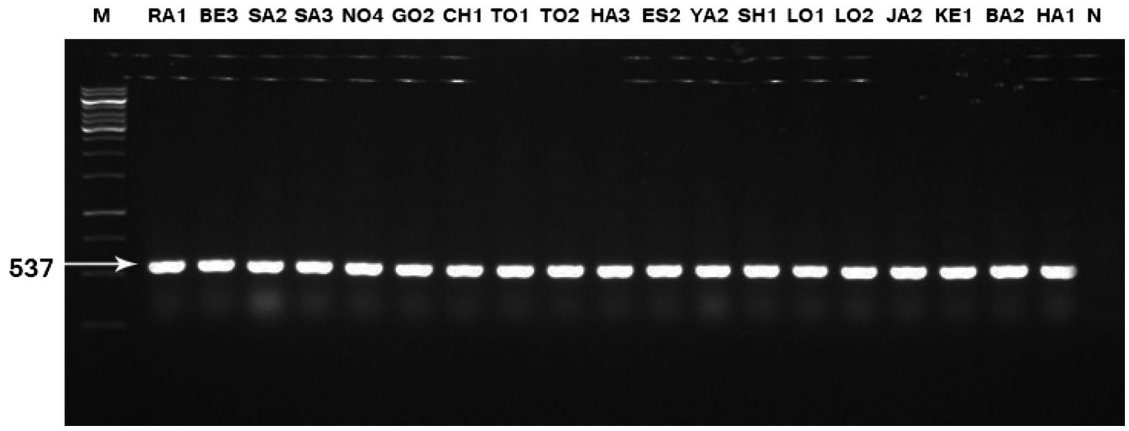

Figure 1. The amplified bands pattern of *B. rubrifaciens* isolates using primers BrAR and BrAF M (GeneRuler 1 kb DNA Ladder), N (Negative control).

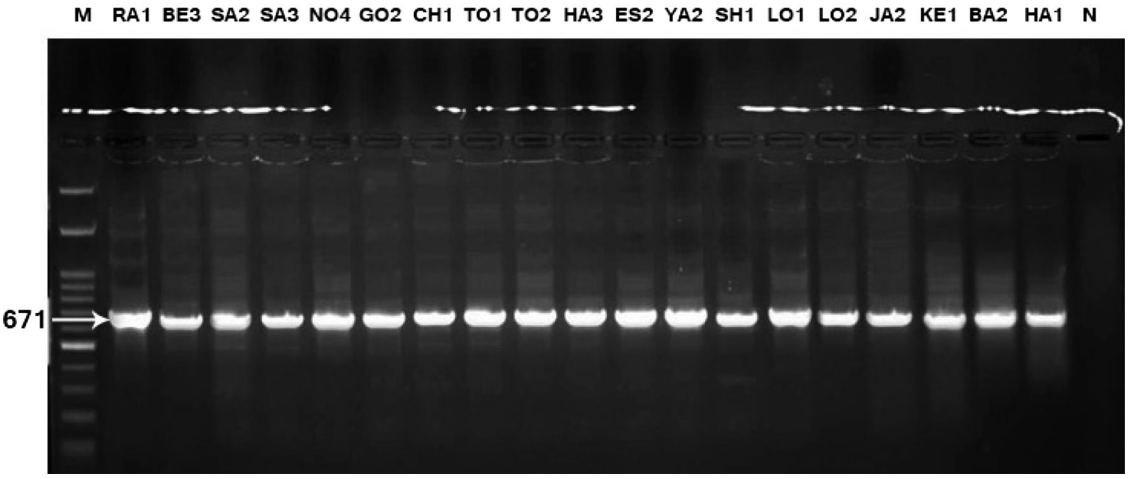

Figure 2. The amplified bands pattern of *B. rubrifaciens* isolates using primers 2BrIF and 2BrIR M (Standard molecular marker 100 bp DNA Ladder), N (Negative control).

difference was observed between them. In the negative control, such a band was not observed that indicated the reaction specificity and improper primers functioning from the viewpoint of reaction occurrence lack was non-specific (Figures 1 and 2).

The disease symptoms on inoculated sapling and fruits

In pathogenicity test on walnut sapling, after 12 weeks, some sings in from of necrosis points, water-burnt spots and black-colored regions were observed, in some cases associated with latex discharge. In addition, no sing was observed in the branches inoculated with sterile distilled water. In case of pathogenicity test on immature walnut fruit, the disease symptoms considering inoculated suspension concentration were different. In the fruits that were inoculated with 10^8 cfu/ml concentration, the disease symptoms in the form of fruit rot along with black-colored and necrosis regions were observed that in some cases was associated with a black-colored latex discharge. The fruits inoculated with a concentration lower than 10^8 cfu/ml, the symptoms only appeared as black-colored small points. In 10^2, 10^3 and 10^4 cfu/ml concentrations, no symptoms were observed on immature walnut fruits. This research was the prime report on pathogenicity of *B. rubrifaciens* bacteria on walnut fruit in the world (Figure 3).

The fingerprinting results of *B. rubrifaciens* isolates on the basis of BOX-PCR

In this investigation, representatives related to isolates of different regions of country were compared. The fingerprinting results of DNA with BOXAIR primer was specified in 13 bands that approximately were located between 300 and 3500 bp (Figure 4).

The similarities between isolates with BOXAIR primer were shown at a domain of 1–82%. The results of cluster analysis with this starter showed that in the similarity level of 82%, the isolates were divided into two genotypic groups. The first group that had the highest pathogen population in it at 93% similarity level was divided into the two following groups that below the first group included the strains related to northern regions of the country and margins of the Caspian sea and below the second group was related to the strains pertained to central and west of the country. The second group created an independent group in relation to the other groups and included the strains of Fars and Kohgiluyeh and Boyer-Ahmad provinces.

The identified haplotypes in each of the groups were specified using the aforesaid primer in Figure 5. The haplotypes were determined at the similarity level of 99% and the strains located in a haplotype group had the highest rate of genetical similarity among the strains located in a main group. Based on the strains located in a haplotype group had 100%

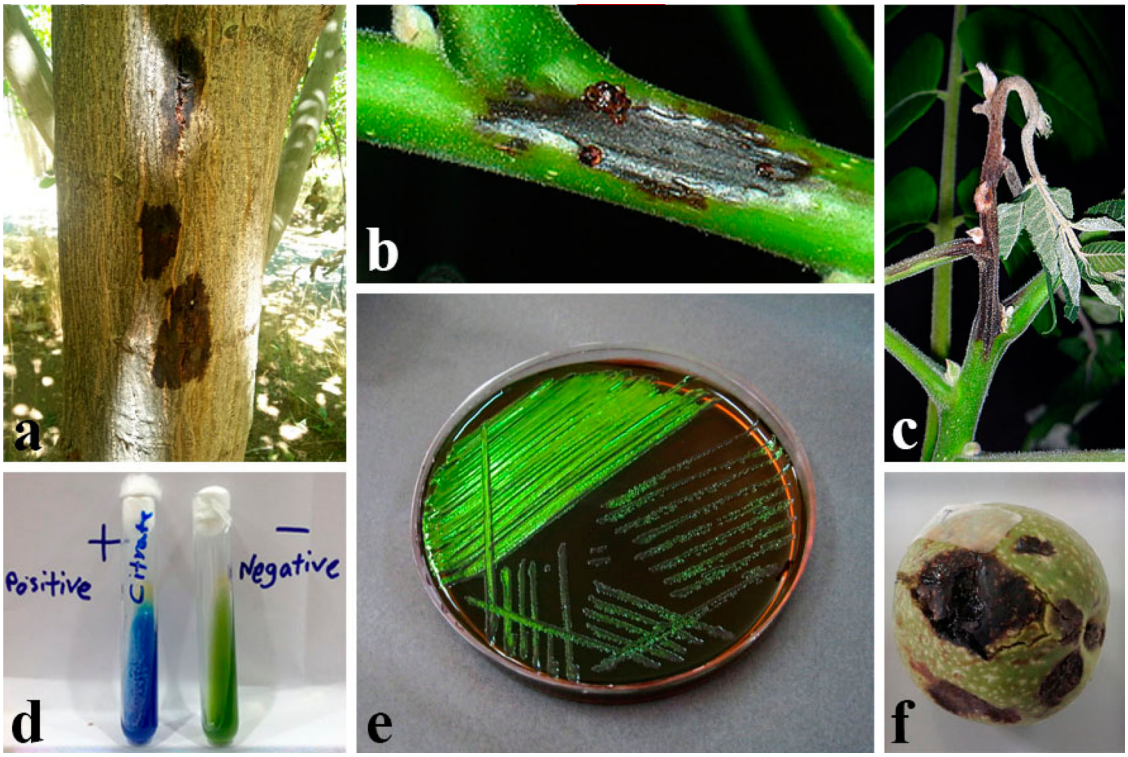

Figure 3. The disease, phenotypic and pathogenicity symptoms of *B. rubrifaciens*. (a) The deep canker symptoms on walnut trunk, (b,c) the sapling inoculated with *B. rubrifaciens* 10^8 cfu/ml suspension three months after contamination, (d,e) the created symptoms in citrate test and growth on EMB media, (f) raw walnut fruit inoculated with bacterial suspension 12 days after contamination with 10^8 cfu/ml concentration.

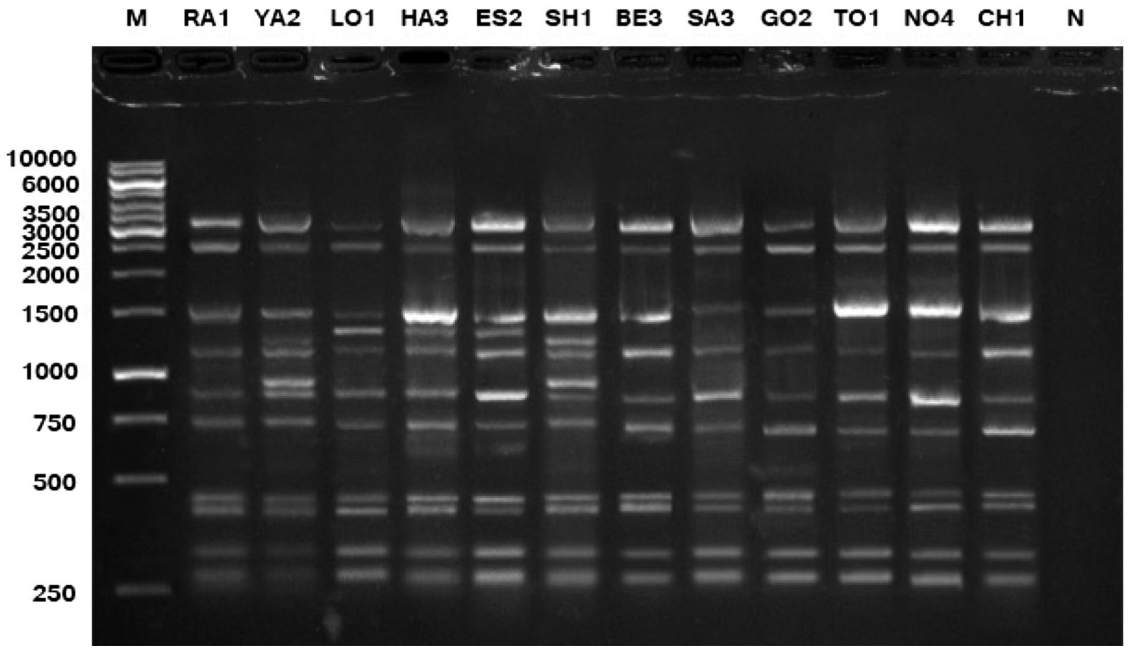

Figure 4. The BOX-PCR pattern of strains, that is, agent of deep trunk canker in Lorestan Province using a primer, M (GeneRuler 1 kb DNA Ladder).

genetical similarity and alike pattern of DNA fingerprinting, in a manner that the entire replicated 13 bands with BOXAIR primer for these strains were similar. In general, the molecular indicator BOX-PCR could very well exhibit the genetic correlation of isolates, which helps in separating them from one another (Table 3).

Discussion

Deep bark canker of walnut trees is caused by the bacterium, *B. rubrifaciens*. The disease is characterized by a chronic and debilitating decrease in yield and tree vigor. Symptoms of deep bark canker more often appear in trees aged at least 10 years. The symptoms include the development of deep longitudinal cracks in trunks, scaffolds and larger branches, which exude a dark-colored mixture of bacteria-laden sap. Previous research suggests that *B. rubrifaciens* may reside in the vascular tissue of trees, where it can lay dormant until a change in environmental conditions (such as water stress) occurs, resulting in the emergence of virulent bacteria and development of disease (McClean and Kluepfel 2008).

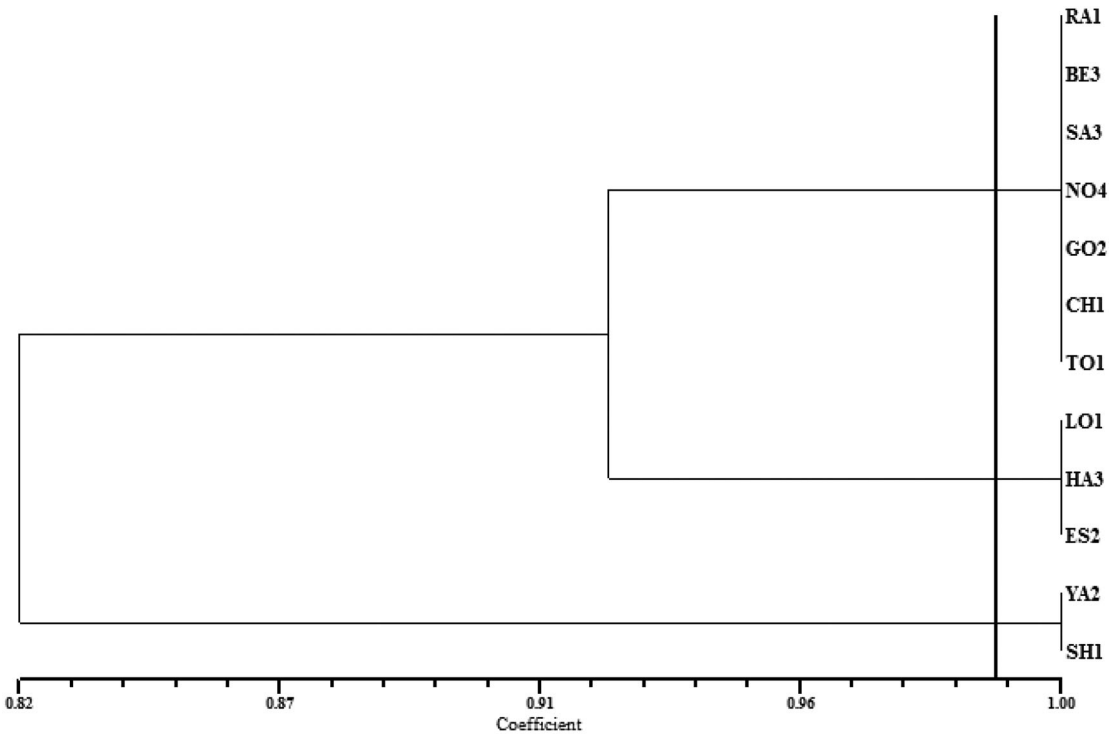

Figure 5. Dendrogram of *B. rubrifaciens* isolates of walnut deep canker based on BOX primer; the line drawn in 99% similarity level, wherein each of the intersecting dendrogram lines indicate the haplotype numbers in each group.

Table 3. The similarity matrix of 13 *B. rubrifaciens* isolates obtained from BOX indicator.

	RA1	YA2	LO1	HA3	ES2	SH1	BE3	SA3	GO2	TO1	NO4	CH1
RA1	1.00											
YA2	0.85	1.00										
LO1	0.92	0.77	1.00									
HA3	0.92	0.77	1.00	1.00								
ES2	0.92	0.77	1.00	1.00	1.00							
SH1	0.85	1.00	0.77	0.77	0.77	1						
BE3	1.00	0.85	0.92	0.92	0.92	0.85	1.00					
SA3	1.00	0.85	0.92	0.92	0.92	0.85	1.00	1.00				
GO2	1.00	0.85	0.92	0.92	0.92	0.85	1.00	1.00	1.00			
TO1	1.00	0.85	0.92	0.92	0.92	0.85	1.00	1.00	1.00	1.00		
NO4	1.00	0.85	0.92	0.92	0.92	0.85	1.00	1.00	1.00	1.00	1.00	
CH1	1.00	0.85	0.92	0.92	0.92	0.85	1.00	1.00	1.00	1.00	1.00	1.00

During the revisits from walnut orchards of different regions of the country, the samples suspected to possess deep trunk canker symptoms were collected. With conduction of phenotypic tests, 28 bacterial isolates of *B. rubrifaciens* were identified, wherein results of polymerase chain reaction validated the results of phenotypic tests. In order to assess the pathogenicity properties, the identified isolates were inoculated to immature fruit and two-year-old saplings of walnut. The formed symptoms on immature fruit appeared as necrosis regions and dead tissue and symptoms on sapling was in the form of black color regions along with latex discharge.

In a research, researchers assessed the biochemical, morphological and physiological characteristics of *B. rubrifaciens* isolates. The results of some of the characteristics include negativity of gram and oxidase reaction, positivity of catalase and anaerobic fermentation (OF positive), ability to produce sulfide hydrogen gas from python and inability to produce endospore and production of sulfide hydrogen gas from glucose and positivity of the movement test. It was well shown that the aforesaid bacterium was devoid of the needed ability to hydrolyze starch, hydrolyze Tween 20 and produce indol, but it was able to grow at 36°C and produce metallic green pigment on EMB . These findings were in concurrence with the results obtained in the present research.

Considering the fact that in this research the entire isolates of *B. rubrifaciens* on YDC medium produced pink pigment, this peculiarity can be used for distinction of this species with *Brenneria nigrifluens* species. In a research, the researchers with an investigation of gene-producing pigment of *rubrifaciens* strived to transfer this gene to *B. nigrifluens* and *Brenneria salicis* strains and eventually the gene producing this pigment did not appear in the case study bacteria and none of the bacteria were able to produce the pigment (Mcclean and Kuepfer 2009). Therefore, the present peculiarity can be used to distinct this species with the other species of *Brenneria*.

A comparison of physiological and biochemical characteristics of standard *B. rubrifaciens* NCPPB 2020T and *B. nigrifluens* NCPPB 564T strains showed that *B. rubrifaciens* NCPPB 2020T strain in relation to acid production from raffinose and rhamnose showed a negative reaction and in relation to esculin hydrolysis and acid production from arabinose showed a positive reaction (Biosca and López 2012); this

is in a manner that approximately 10% of the present research isolates in relation to the raffinose and rhamnose tests showed positive and in relation to esculin and acid production from arabinose showed a negative reaction.

In revisits of walnut orchards, deep trunk canker symptoms were observed in the form of darkening of the outer layer of skin and in summer in the form of latex exit from the trunk of walnut trees wherein the findings of other researchers validate symptom identification case of disease (Giorcelli and Gennaro 2014). In the polymerase chain reaction using specific primers, the genetic templates related to asparagine synthetase gene with an extension of 537 bp and autoinducer synthase gene with an extension of 671 bp were amplified that had conformity with the size of amplified template in the results of Tapa et al. For molecular identification of disease agent in addition to primers BrAF/BrAR and 2BrIF/2BrIR, the specific primer pair BR1/BR3 that was designed based on a specific sequence in 16SrDNA genome in PCR reaction led to the production of a template with an extension of 409 bp. The primer BR was specifically used for identification of *B. rubrifaciens*, whereas in other bacteria does not lead to sequence amplification and production of genetic template (McClean et al. 2008).

From the historical viewpoint, independent methods of DNA, such as physiological and biochemical tests, and protein map were used for the characteristics' identification and phenotypic study of variation between the Enterobacteriaceae. The techniques that assess variation in genomic DNA have created powerful tools with repeatability and higher assurance for the variation assessment. The DNA fingerprinting based on the presence of repetitive sections that are present diffusely in the genome of bacteria (BOX-PCR) is an assured and sensitive method for the assessment of variation in species, sub-species and strain level. The investigation of genetic variation in herbal pathogen population improves the taxonomy identification, epidemiology and its diagnosis (Marefat 2005). In a research to conduct DNA printing for 12 isolates of *B. rubrifaciens* bacteria, the BOX-PCR technique and BOXAIR primer was used. The results of this research showed 100% polymorphism among the strains and templates of sizes 150–3000 bp were amplified. These templates had a total conflict with produced size of the templates in this research, which was in the range of 300–3500 bp (Giorcelli and Gennaro 2014). These genetical differences among strains can be due to different molecular mechanisms such as mutation in DNA due to disorder in the DNA replication process. This possibility exists that each of the mechanisms must have taken place in deep trunk canker and led to the creation of genetic variation levels among *B. rubrifaciens* population, or epinephrine on some of the hosts without creation of symptoms could help in the formation of genetical variation in the pathogen population (Choi et al. 2000). The researchers use other methods, namely RAPD (Random Amplification of Polymorphic DNA), RFLP (Restriction fragment length polymorphism) techniques for achievement of genetic variation among the herbal pathogenic bacterial population. The RAPD indicator due to the use of short primers with low connection temperature has a low repeatability and this method is much influenced by the environmental conditions. Considering that in RFLP method a section of the genome is assessed, it is possible that in some cases one cannot distinguish the isolates. However, one of the distinctive advantages of BOX-PCR method compared to other methods, is being based on much protected repetitive sequences. This method is used a fast, precise and repeatable method for identification and genetically investigation of different bacterial sections (Harighi and Roshangar 2009).

Considering the conducted revisits in 2015 in some of the orchards whereby sample collection was conducted and presence of *B. rubrifaciens* bacteria was proved, it was determined that initiation of disease progressions each year generally appears from the canker locale of the previous year, and therefore the probability is high that bacteria hibernate in the superficial wood tissue and under skin layer.

With the investigation of walnut tree in 2015 in the laboratory, no bacteria of this type were obtained. The probable regions for the bacteria staying alive, are under the skin of the trees, and the soil beneath the trees, this requires more studies.

The trees that in any mode are exposed to unsuitable environmental stresses, such as irrigation stress and or insufficient nutrition, sooner and higher suffer from this disease. Meanwhile, some of the trees wherein their trunk and branch are affected by wound and scratch due to attacks of birds, insects, human or other mechanical factors suffer from deep canker and fungal canker. It seems that wound formation in trunk and branch tissues is one of the needed and essential routes for permeation of bacteria and other pathogenic factors. Since the sick trees cannot be cured finding resistant genotypes is necessary, it is suggested to use healthy and non-contaminated saplings in an orchard establishment. The irrigation of walnut orchards should be done in a balanced way, and in case of possibility the drip irrigation method must be used. In facilities that lack this type of irrigation, basin irrigation (instead of flood irrigation) of orchards should be used. In case of contamination outbreak, the cutting of branches, offshoots and dried sections of diseased trees should be carried out a bit far from the infection locales; besides, scraping of trunk cankers in contamination locale and immediate fumigation of trees with suitable concentration of copper poisons (copper oxychloride or Bordeaux mixture) and bandage of wounds locale with orchard tape two to three times at an interval of 20 days are effective measures to prevent advancement and severity of disease and delay death of diseased trees.

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