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Isolation and Characterization of *Brenneria nigrifluens* Causing Bacterial Shallow Bark Canker of Walnut Trees in Golestan Province, Iran

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ABSTRACT

Shallow bark canker incited by *Brenneria nigrifluens* is considered one of the most dangerous diseases which can cause a significant reduction in walnut and timber production. During spring and summer of 2019, walnut gardens were surveyed for symptoms like leaf spot, fruit, and stem canker from four different regions of Golestan province located in Iran. A set of 11 Gram-negative bacteria were isolated from sample tissues with symptoms. In pathogenicity tests on unripe walnut fruits, all isolates caused typical black necrotic lesions covering almost the entire pericarp. Results of selected phenotypic tests and whole-cell protein patterns indicated that all isolates were the same as described for the type strain of *B. nigrifluens*. Phylogenetic analysis based on sequencing of the *gyrB, rec A,* and *16s rRNA* genes of the representative isolate formed a unique clade, well-characterized, and separated from related species. The results of this study can have a bearing on the choice of disease management strategies.

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Introduction

The causal agent of shallow bark canker of Persian walnut trees, B. nigrifluens, has become fairly widespread in Iran in recent years. Early stages of the disease are not evident due to the presence of swollen areas on the surface of the skin, which is often superficial on the skin, and gradually shows dark brown leachate with scares (Moretti *et al*., 2007). Extensive necrosis of the underlying tissues could be observed by removing phelloderm (Piccirillo, 2003). Phenotypic and molecular characteristics showed genetic diversity among pathogen populations using the BOX-PCR method (Amirsardari *et al*., 2017; Sadeghi and Khodakaramian, 2020). Although the disease causes more damage in the rainy regions, the agent is not just restricted to the rainy and humid climates (Hauben *et al*., 1998; Saccardi *et al*., 1998a; Jamalizadeh *et al*., 2009). At first, the disease was reported in California (Wilson *et al*., 1957), then in Spain (Lopez *et al*., 1994), Italy (Saccardi *et al*., 1998b; Morone *et al*., 1998, Scortichini, 1999, Carella *et al*., 2003; Loreti *et al*., 2005), Korea (Choi *et al*., 2000), France (Menard *et al*., 2004), and other provinces of Iran (Rahimian, 1989, Harighi and Rahimian, 1997; Baradaran and Ghasemi, 2004; YousefiKopaei *et al*., 2004; Harighi, 2006; Jamalzade *et al*., 2009; Amirsardari *et al*., 2015). Recently, symptoms of the disease have been observed in a walnut garden located in different regions of Golestan province, Northeast of Iran. Therefore, this study aimed to identify and characterize the causal agent of the disease in Golestan province, Iran.

Materials and Methods

Sampling and bacterial isolation

During 2019, shallow canker symptoms were observed in the walnut gardens in different regions of Golestan province, located in Northeast of Iran. Samples were collected and transferred in plastic bags to the laboratory. To isolate the causal agent, samples with symptoms washed with sterilized-tap water carefully, disinfested with sodium hypochlorite 1% for one minute followed by rinsing three times in sterilized distilled water. Small pieces of tissues were cut and placed in 100 mL PBS (Phosphate Buffered Saline) sterilized distilled water. The suspension streaked on eosin methylene blue agar medium (EMB) (Loreti *et al*., 2008) for colony appearance. Plates were incubated at 28°C, monitored for 2-4 days. Single bright green metallic colonies from the EMB medium were selected and streaked on nutrient agar (NA) as pure single colonies.

Hypersensitivity and pathogenicity test

Hypersensitivity reaction (HR) test on Geranium leaves was performed using bacterial suspension from a fresh culture on the NA medium with a concentration of 10^8 CFU/ml (OD_{600} = 0.1). Leaves were evaluated 24 and 48 hours after inoculation (Klement *et al*., 1964). To validate the pathogenicity of isolates, the pathogenicity test of bacterial isolates on immature walnut fruits was performed by Moretti and Buonaurio, (2010) method. Immature fruits were first disinfected with 70% alcohol. Pure bacteria isolates were grown on nutrient agar at 27 °C for 48 h. The bacterial suspension was adjusted to OD600 = 0.1 (ca. 10^8 CFU/mL) with 0.01 M Magnesium sulfate (pH 7.2), further diluted to 10^6 CFU/mL, injected into the immature walnut fruits mesocarp layer with a syringe, then kept at the desired temperature and humidity chamber. Magnesium sulfate buffer was used as negative control injected into some fruits. The pathogenicity tests were conducted twice. To fulfill the pathogenicity test, the same bacterial strains were isolated successfully.

Protein analysis through SDS-PAGE

Electrophoresis of soluble proteins was carried out in a discontinuous SDS polyacrylamide gel according to the method of Laemmli (1970). For each culture, a loopful of 24-hour growth from the NA plate was suspended in 50 ml King's B broth and incubated in a rotating incubator for 16 h (at 27 °C, 150 rpm). The samples were then transferred into Eppendorf tubes and centrifuged for 5 minutes at 10000 × g. The collected cells were washed three times with sterile distilled water. The washed cells were stirred after the addition of 25 µl SDS sample buffer (0.06 M Tris, 2.5% Glycerol, 0.5% SDS, 1.25% β-mercaptoethanol, and 0.001% bromophenol blue) and the proteins were denatured in boiling water for 5 minutes. The supernatant was then centrifuged again for 5 minutes at 10000 ×g, collected in an Eppendorf tube, and kept at -50 °C until electrophoresis was carried out. Fifty microliters of soluble proteins were loaded in each well in a 13 × 17 cm polyacrylamide slab with 0.75 mm thickness. Proteins were fractionated in 10% resolving gel at a constant current of 20 mAmps for 4 h. The gel was stained in methanol, water, and acetic acid (5:5:1) containing 0.5% coomassie brilliant blue G250 overnight and destained in the same solution without dye. The gel was kept in 7% acetic acid.

Biochemical and phenotypic characteristics

Biochemical and morphological tests were carried out in duplicate according to the standard bacteriological criteria. The tests were as follows: oxidase and gram reaction (Suslow *et al*., 1982 and Schaad *et al*., 2001), production of pigment fluorescent on King-B medium (King *et al*., 1954), levan production, arginine dehydrolase (Lelliot *et al*., 1966), catalase, starch hydrolysis, Esculin, Tween 80, Gelatin hydrolysis, oxidation-fermentation of glucose, growth at 37 °C (Fahy and Persley, 1983), Nitrate reduction, growth in 5% NaCl (Schaad *et al*., 2001), utilization of carbon and nitrogen sources (Schaad *et al*., 2001). Extra tests are added in table 1.

DNA extraction and gene amplification

Genomic DNA extraction was performed by the chloroform-isoamyl alcohol method as described by Hu *et al*. (2007). The quality and yield of DNA were assessed by using a spectrophotometer (Eppendorf BioPhotometer plus) or by the gel electrophoresis. The *16S rRNA* gene was amplified using fD1 (5´CCGAATTCGTCGACAACAGAGTTTGAT

CCTGGCTCAG-3´) and rP2 (5'ACGGCTACCTTGTTACGACTT 3') primers (Weisberg, 1991). These primers are designed to yield nearly full-length *16S rRNA* genes from most bacteria. Also, two housekeeping genes (*recA* and *gyrB*) were amplified based on *recA* (Waleron *et al*. 2002) and *gyrB* (Popovic *et al*., 2013) primers, respectively. PCR amplification was performed in a thermal cycler (MJ MiniTM Gradient Thermal Cycler) with 50 µL total volume containing 2 µL of each primer (10 µM), 5 µl of 10x prime *Taq* Reaction Buffer (GenBio, South Korea), 2 µL of dNTP (2.5mM each), 6 µL of 25mM Magnesium chloride, 0.3 µL of Prime *Taq* DNA Polymerase (5 units/µl, GenetBio), 2 µl of template DNA (~500 ng/µLin concentration) and 30.4 µL sterile purified water (Mili-Q Water). The cycling conditions were 5 min at 94 °C, 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 90 s, and final elongation at 72 °C for 10 min.

Sequencing and DNA analysis

The PCR products were run on 1% agarose gels in 1.0× Tris-acetic acid-EDTA (TAE) buffer and the target fragments (expected-size bands) were excised and cleaned according to the manufacturer's protocol of GF-1 AmbiClean Kit (Vivantis, Malaysia). The PCR products were sequenced in both directions using the PCR primers in the Macrogen Company (Humanizing Genomics, Macrogen, South Korea). The nucleotide sequences were compared with others in the GenBank database using the BLASTn program. Multiple sequence alignments were performed by Clustal W (Thompson *et al*., 1997) and phylogeny was constructed by the neighbor-joining method at 1000 bootstrap (Saitou and Nei, 1987). The phylogenetic trees and molecular analysis were carried out using the MEGA version 7.0 software "Mega, Molecular Evolutionary Genetics Analysis, Pennsylvania, USA" (Tamura *et al*., 2011).

Results

Bacterial isolation

A total of 11 isolates were recovered from the samples showing symptoms of canker and dieback (Fig. 1) in infected branches of walnut trees in Golestan province (Minoodasht, Gonbad Kavus, Negin-Shahr, and Azad-Shahr).

The initial classification of collected bacterial strains was performed by Gram reaction, color, and form of colonies. All strains were grouped based on whole-cell bacterial protein patterns through the polyacrylamide gel electrophoresis method (SDS-PAGE) (Fig. 2).

Fig. 1. The shallow bark canker and deep necrosis Symptoms: A) The symptom of shallow bark canker on the stem. B) The symptom of deep necrosis after removing branch bark.

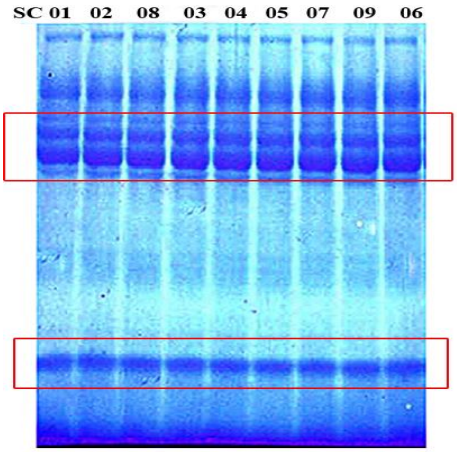

Fig. 2. SDS-PAGE of whole-cell bacterial protein showing an identical pattern in whole samples studied.

Based on whole-cell protein analysis, all isolates showed an identical pattern and were grouped in the same group. Subsequently, one representative strain was selected from the same protein patterns for further investigations. The

representative was subjected to the biochemical and phenotypic tests, evaluated based on standard bacteriological keys, and compared with the type strain of *B. nigrifluens* (Table 1).

Table.1. Biochemical and phenotypic tests of isolates used in this study compared with the type strain.

Test	Bacterial isolates	*B. nigrifluens (Type strain)*
Urease	+	V
Oxidative / Fermentative (O/F)	+	+
Catalase	+	+
Oxidase	-	-
Indole production	-	-
Pink colony on YDC	-	-
Levan	-	-
Starch hydrolysis	-	-
Arginine dihydrolase	-	-
Oxidative	+	+
Fermentative		
Growth in 5% NaCl	+	+
Growth at 37°C	+	+
Tween 80	-	nd
Gelatin	-	-
Esculin	+	+
Casein	-	-
Lecithin	-	nd
Acid Production of :		
Maltose	-	-
Cellobiose	+	nd
Glucose	+	+
Galactose	+	+
Raffinose	+	+
Lactose	-	-
Mannitol	+	+
Xylose	+	+
Fructose	+	+
Glycerole	+	+
Utilization of carbon sources:		
Propionate	-	-
Tartrate	-	nd
Malonate	-	-

+: 85% or more positive; -: 85% or more negative; nd: not determined; V: variable. The experiments were conducted twice, Hauben and Swings, (2005).

Hypersensitivity and pathogenicity

The isolates did not induce a hypersensitive reaction (HR) on geranium leaves after 12-24 hours following the injection of the bacterial suspensions. In the pathogenicity test, the water-soaked spot appeared on immature walnut fruit three days after inoculation. Water-soaked symptoms developed and turned to necrosis with brown to black exudates on the inoculated fruits. Bacterial colonies with the same phenotypic characteristics as originally described were re-isolated from the artificially inoculated fruits. No symptoms developed on control fruits which injected by Magnesium sulfate buffer (Fig. 3).

Fig. 3. Production of water-soaking after bacterial inoculation to mesocarp of immature walnut fruit (both fruits in the left) and negative control by Magnesium sulfate buffer which injected into the single right one.

Phylogenetic analysis based on *16S rRNA*, *recA* and *gyrB* genes

The representative strain was subjected to molecular studies. To determine the genus of the bacterial isolates, we sequenced the *16S rRNA* gene. The obtained sequences were deposited in GenBank (MT235539). In a first step, the sequences were BLASTed against NCBI's *16S rRNA* GenBank. Querying the representative strain sequence revealed a 99.9% sequence identity with the strain *B. nigrifluens* (Accession No, NR 026048.1), meaning that the isolate might belong to that species. On the other hand, the phylogenetic tree by the neighbor-joining method (Fig. 4) shows that the representative strain as part of the *B. nigrifluens* lineage. However, these results are insufficient to conclude. To expand on the *16S rRNA* gene analysis results, we partially sequenced the two housekeeping genes, *recA*, and *gyrB*.

Each one of these genes was then BLASTed against the GenBank nucleotide database (NCBI). BLAST analysis showed that all the sequences of the two genes had sequence identities of 99%–100% with those of *B. nigrifluens* reference strains. Furthermore, the phylogenetic analysis using the *recA* (GenBank MT517276) and *gyrB* (GenBank MT510923) genes sequences formed a unique clade, well-characterized, and separated from related species (Figs. 5 and 6).

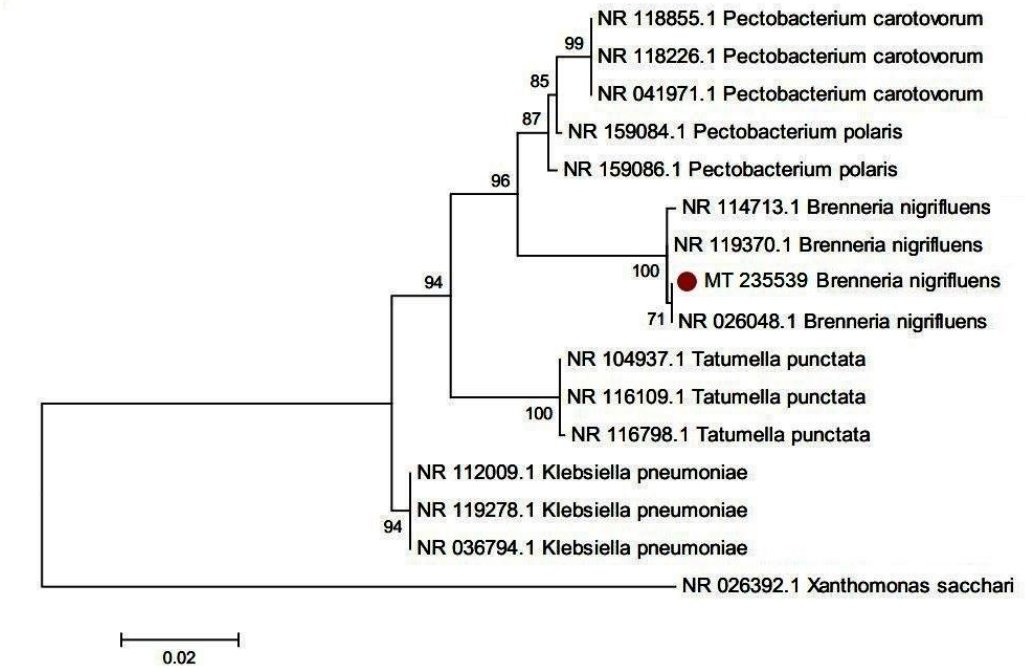

Fig. 4. Phylogenetic analysis of representative strain and related species by the neighbor-joining method based on *16S rRNA* gene sequences: The GenBank accession number for each microorganism used in the analysis is shown behind the species name. Bootstrap values are shown at the nodes. The tree was rooted with *Xanthomonas sacchari*.

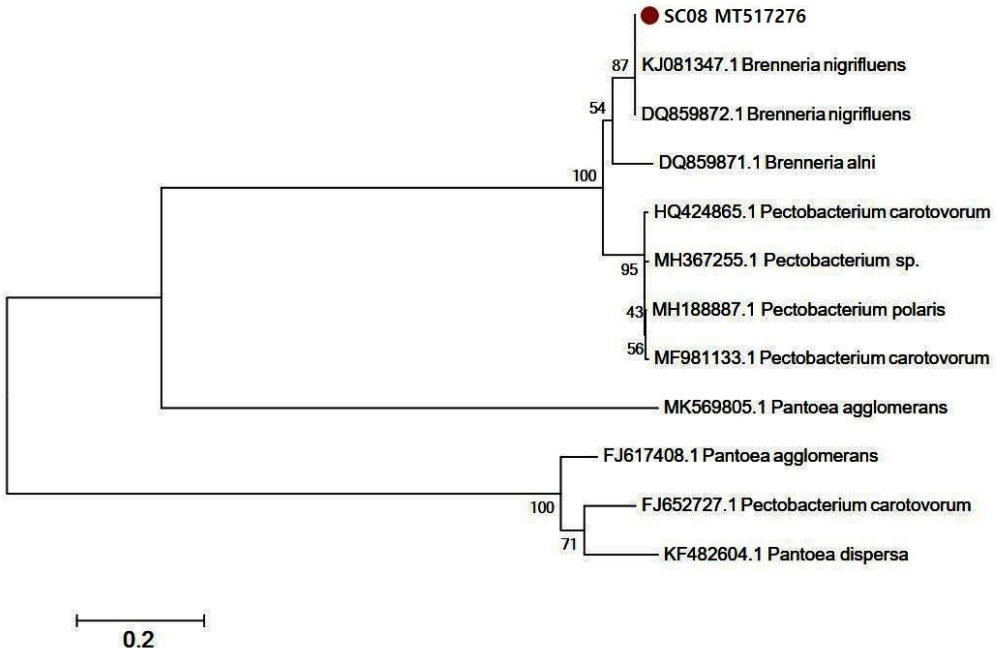

Fig. 5. Phylogenetic analysis of representative strain and related species by the neighborjJoining method based on recA gene sequences: The GenBank accession number for each microorganism used in the analysis is shown behind the species name. Bootstrap values are shown at the nodes.

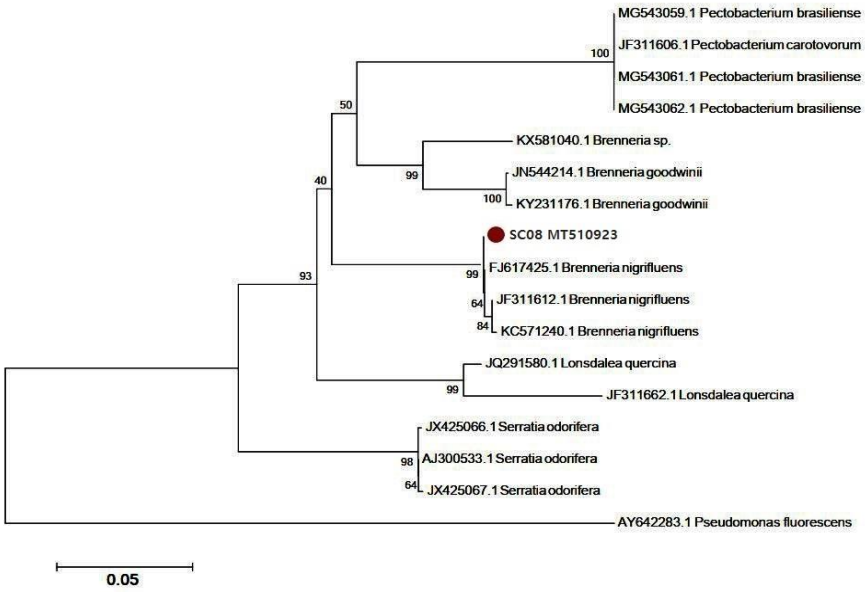

Fig. 6. Phylogenetic analysis of representative strain and related species by the Neighbor-Joining method based on gyrB gene sequences: The GenBank accession number for each microorganism used in the analysis is shown behind the species name. Bootstrap values are shown at the nodes. The tree was rooted with *Pseudomonas fluorescens*.

Discussion

During the past few years with an increase in the cultivation level of walnut trees in Iran, the symptoms of bacterial diseases of walnut in orchards have increased. In this research, the causal agent of shallow bark canker of walnut in the Golestan province of Iran was characterized. Based on morphological, phenotypic, nutritional, and molecular characteristics, we identified the causal agent of shallow bark canker of walnut as *B. nigrifluens*. In this regard, studies have been performed in some provinces in Iran including Mazandaran (Charkhabi *et al*., 2010), Kurdistan (Roshangar and Harighi, 2009), Kerman (Sadeghi *et al*., 2016a; Charkhabi *et al*., 2010), Lorestan, Hamedan, Esfahan, and Shiraz (Amirsardari *et al*., 2017; Charkhabi *et al*., 2010). Furthermore, *B. nigrifluens* strains demonstrated the diversity based on the areas of collection, physiological, biochemical characteristics, electrophoretic patterns of whole-cell protein, and PCR generated DNA fingerprints. The research conducted by Jamalizadeh *et al*. (2009) showed that less than 15% of *B. nigrifluens* strains from the north of Iran were different in some characteristics. Harighi and Rahimian (1997) have demonstrated the existence of some variation in whole-cell proteins electrophoretic profile of strains, isolated from Northern provinces of Iran. However, the current study has found that the collected isolates from four different regions of Golestan province were similar at their whole-cell protein patterns. In reviewing the literature, characterization based on electrophoretic protein patterns of SDS-PAGE correlated closely with the genotyping results (Aeini and khodakaramian, 2017; Vauterin *et al*., 1990). The isolates were tentatively grouped and documented based on polyacrylamide gel electrophoresis in one group. The findings of the current study are consistent with those of Roshangar and Harighi, (2009) who found, the isolates of *B. nigrifluens* collected from Northern provinces of Iran had similarities in their protein pattern and phenotypic characteristics. Moretti and Buonaurio, (2010) described the reliable, reproducible, rapid, and specific test for the pathogenicity instead of the long time process of pathogenicity test on seedlings, which the canker symptoms do not appear until at least one month after inoculation. According to their findings, we examined this test on the mesocarp of immature walnuts which saved time and space compared with the plant stem inoculation technique. To identify the bacteria on the molecular basis, *recA* gene sequences were

analyzed. Sadeghi *et al*. (2016b) also, recognized *B. nigrifluens*, collected from Alborz walnut gardens, based on this housekeeping gene. Another housekeeping gene was *gyrB*, which confirmed this bacterium as *B. nigrifluens* on walnut trees. The *gyrB* housekeeping gene has been used to identify shallow bark canker of walnut in some researches (Popovic *et al*., 2013; Allahverdipour *et al*., 2020).

In conclusion, this study identified and characterized bacterial shallow bark canker on walnut in the Golestan province of Iran. The results of this study can have a bearing on the choice of disease management strategies.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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