گزارش علمی کوتاه

باکتری *Brenneria nigrifluens*، تهدیدی برای گردوی استان البرز

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بیماری شانکر سطحی پوست گردوی ایرانی (*Juglans regia* L.) با عامل *Brenneria nigrifluens* یکی از بیماری‌های مهم و خسارت‌زا روی تنه و شاخه‌های اصلی درختان گردو می‌باشد. تشخیص به‌موقع عامل بیماری، نقش مهمی در جلوگیری از خسارت‌های جبران‌ناپذیر آن دارد. در تابستان سال ۱۳۹۲، تعدادی جدایه باکتری از درختان گردو در استان البرز جداسازی گردید. این درختان علایم آشکاری از شانکر پوستی را نشان می دادند. براساس مطالعات فنوتیپی و ژنوتیپی عامل بیماری باکتری *B. nigrifluens* تشخیص داده شد. این نخستین گزارش رسمی از وجود این بیماری در استان البرز ایران است. وجود این بیماری در استان البرز می تواند خطری نهفته برای گردوکاری‌های گسترده این استان باشد.

کلیدواژه: گردوی ایرانی، شانکر سطحی پوستی، استان البرز

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Brenneria nigrifluens, a latent threat for walnut trees in Alborz province of Iran

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In the summer of 2013, some bacterial isolates were obtained from walnut tissues with the symptoms of shallow bark canker in the Alborz province of Iran. Based on the phenotype and genotype features, the causal agent was identified as *Brenneria nigrifluens*. It was the first report of *B. nigrifluens* causing shallow-bark canker in walnuts in the Alborz province of Iran. The existence of this disease in the Alborz province can pose a latent threat to many walnut orchards in that province.

In Iran, Persian or Black walnuts (*Juglans regia* L.) are regarded as the most important trees for their edible nuts and have hardwood timbers of a high quality. Shallow bark canker is one of the most serious diseases of walnut trees in some countries, like Italy, Spain, and France (Cabello *et al.* 2016, Falahi Charkhabi *et al.* 2010). In Iran, the existence of shallow bark canker in walnuts has been reported in various regions, including the provinces of Mazandaran, Kerman, Kurdistan, Guilan, Golestan, Fars, and Kohgiluyeh-va-Boyerahmad (Jamalzade *et al.* 2009, Amirsardari *et al.* 2015, Falahi Charkhabi *et al.* 2010, Yousefikopaei *et al.* 2007). The bacterium *Brenneria nigrifluens* (Wilson *et al.* 1957), a member of Pectobacteriaceae, is the causal agent of this type of canker. This disease attacks the trunks and branches of young and mature trees. The symptoms include the dark brown lesion with dark liquid exudates excreted from the canker sores of the bark from late spring to autumn. Although Black walnuts are planted increasingly and managed carefully in Iran, no accurate data is available on the economic losses of walnut trees caused by shallow bark canker. Although, walnut trees are native to the Alborz province, the disease was not detected in this province until recently. In the present study, infected walnut trees were observed for the first time in the Alborz province with the typical lesions of shallow bark canker. The color of the trunks and branches ranged from brown to black with dark brown lesions that exuded a dark liquid through the cankers mainly in late summer. Some small pieces of the symptomatic tissue (the edge of healthy and affected tissues) collected were macerated in the Phosphate-buffered saline and streaked onto the Luria-Bertani medium (Atlas 2005). Plates were incubated at 27°C for 72 hours and prevalent colonies were purified on the Eosin methylene blue medium (Schaad *et al.* 2001). Ten Gram-negative and oxidase-negative strains, showing oxidative-fermentative positive metabolism, were selected for further characterization. Physiological and biochemical tests showed that the strains that grew at 36°C tested negative for the indole, arginine dihydrolase, and nitrate reduction and did not produce H_2S from peptone. The strains did not induce a hypersensitive reaction on tobacco leaves but induced a hypersensitive reaction on geranium leaves after 24 hours; however, they did not hydrolyze gelatin and starch. The pathogenicity of the selected strains was confirmed by

Fig 1. Symptoms of bacterial canker caused by *Brenneria nigrifluens* on walnut (*Juglans regia*) young twigs one month after inoculation.

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Fig 2. Gel electrophoresis analysis of polymerase chain reaction products amplified from the genomic DNA (40 ng) of *Brenneria nigrifluens* strains using specific F1–C3 primers pair. Lane 18a: *B. nigrifluens* lane C+; Psitive control. Lane C-: water negative control. M: molecular marker Gene Ruler 100 bp DNA ladder.

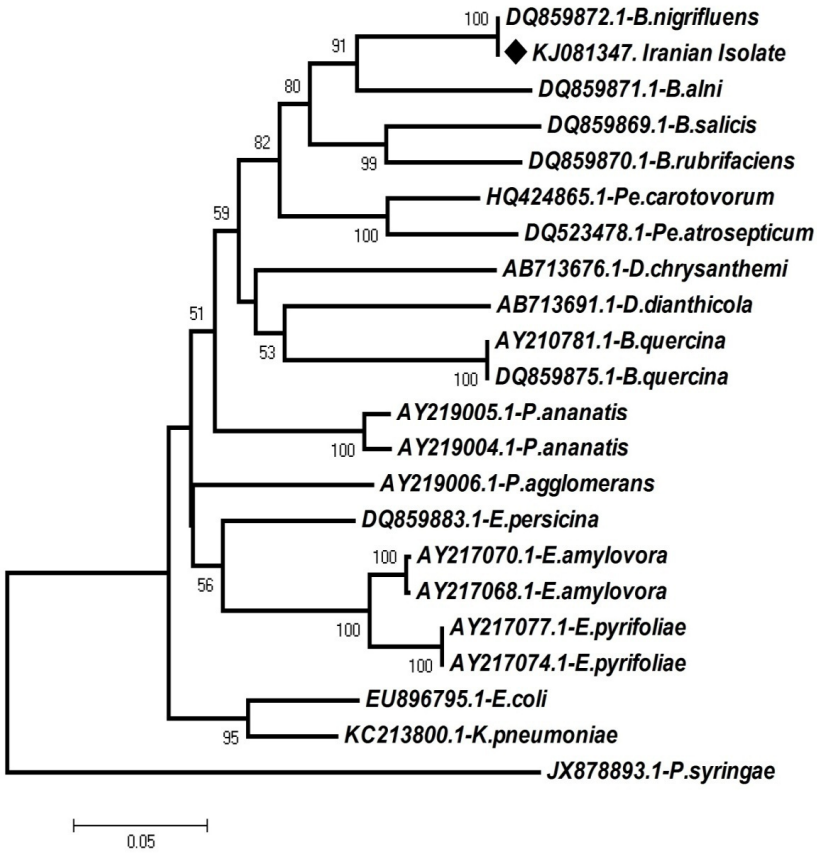

Fig3. Phylogenetic tree derived from neighbor joining analysis of the *recA* genes. Number KJ081347: Iranian *Brenneria nigrifluens* strain; *Pseudomonas syringae* JX878893.1 was used as out group for this analysis.

inoculating the branches of one-year-old walnut trees with 10^9 CFU (based on the turbidity measurement (600nm wavelength)) of the isolate introduced into the wounds. In the same vein, some branches were inoculated with water as controls. One month later, necrotic lesions were observed in the inner bark, and dark lines were observed in the internal wood on the branches inoculated with the bacterium (Fig. 1). No external canker was observed on any branches inoculated with water. The bacterium was re-isolated from the lesions on the shoots and identified as it is described below. The bacterial genomic DNA was extracted by a single phenol-chloroform step, precipitated in 2-propanol, and suspended again in 100µl of sterile distilled water (Ausubel *et al.* 1992). All PCR reactions were performed in the final volumes of 50 µl PCR using the specific oligonucleotides of F1 and C3 (Loreti *et al.* 2008), and the amplification product of the expected size (255 bp) was obtained with the genomic DNA (Fig.2). To identify the bacteria on a molecular basis, *recA* gene sequences were analyzed. The *recA* gene sequences were generated using the primers of recAR and recAF (Waleron *et al.* 2002). All amplicons were purified using the AccPrep PCR purification kit (Bioneer), according to the manufacturer's instructions and sequenced by Macrogen corp. (Seoul, South Korea) through the same primers used for amplification. The sequences were edited using the software Bioedit and assembled using the program ClustalW. Phylogenetic trees were produced using the Tamura-Nei genetic distance model and the Neighbor-joining method (Saitou & Nei 1987) by the software Mega4 (Tamura *et al.* 2007). The r*ecA* sequence of the bacterial strain identified as *B. nigrifluens* revealed a high similarity (100%) to other sequences of *B. nigrifluens* DQ859872.1 in GenBank (Fig. 3). The nucleotide sequences were deposited in the NCBI GenBank nucleotide sequence database (Accession number: KJ081347.1). So far as we know, this is the first report of *B. nigrifluens* in the Alborz province of Iran. One of the isolates used in this study was deposited in the culture collection of the International Collection of Microorganisms from

Plants (ICMP) as *B. nigrifluens* ICMP 20120. The shallow-bark canker of walnuts is the major concern throughout the nut-producing regions of Iran. It results in significant economic losses, so it is extremely difficult to be controlled. Accurate diagnosis is the most important step in controlling a plant disease. Most often, the control of the disease fails, since the disease is initially misdiagnosed. To ensure safe and sustainable agriculture, the early detection of such harmful bacteria in plants is essential. Furthermore, using rep-PCR techniques, some variations have been observed in the strains of *B. nigrifluens,* and specific fingerprint patterns have been linked to some geographic areas in Iran (Falahi Charkhabi *et al.* 2010). This is likely since Persian walnut cultivation is primarily based on the ecotype, and local seedlings have adapted to a particular environment, making the selection of different *B.nigrifluens* populations possible. The importance of pathogen variation in the effectiveness and durability of host resistance was examined by Rahimian (not published). Pathogen genotypes can interact with specific host genotypes, thereby leading to the "breakdown" of resistance within quite short periods of time (Brown 1996). Detecting the genetic diversity of pathogens is based on the identification of the virulence variation in pathogen populations. This approach to monitoring pathogen populations has contributed to the development and establishment of host resistance and provided important insights into the evolution of pathogen populations in response to the selection by the host resistance (McDonald & Linde 2002). Various walnut ecotypes are cultivated in different regions of Iran, so this study could detect the growth of any new population of the bacterium; thus, it is relevant to the choice of the disease management strategies. Further studies are recommended to be conducted on the implementation of sanitation processes required to reduce the prevalence rate of the disease for canker-free orchards.

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