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Phenotypic and Molecular Characterization of *Neofusicoccum mangiferae*, the Causal Agent of Black Locust Decline

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Abstract

The last few years have seen the rapid and obvious deterioration, wilt, and death of black locust trees in urban and sub-urban areas of Markazi province, Iran. These swift changes are having a serious effect on the landscape and urban forestry. This study was conducted using phenotypic and molecular methods to identify the causal agent of the decline and death of the black locust trees. Pathogen isolation from infested trees was achieved using a potato dextrose agar medium. All the isolates of the fungus produced arthroconidia, making identification using morphological properties possible. *Neofusicoccum mangiferae* was implicated as the cause of the black locust decline via standard taxonomic criteria for diseased trees. In a pathogenicity test that involved the insertion of a mycelial plug of the respective isolates under the bark, similar symptoms were produced as natural infection. Species classification was confirmed by analysis of the internal transcribed spacer (ITS) sequencing.

Keywords: Molecular characterization; Forest; Fungus; Dieback; *Robinia pseudoacacia*

Introduction

Black locust (*Robinia pseudoacacia* L.) is a nitrogen-fixing deciduous tree belonging to the Fabaceae (legume) family [1]. It is native to southeastern North America but has been naturalized in temperate regions around the globe. It is a multi-purpose species suitable for soil erosion control, fuels, land reclamation, and as a nectar source for beekeeping [2]. It is also used for forage, lumber production, and wood fiber [3]. It is a fast-growing tree, resistant to many environmental stresses, e.g., low and high temperatures, drought, and air pollutants, and tolerant of low fertility soils [4]. Black locust can be attacked by several fungi, bacteria, and viruses, such as *Phomopsis oncostoma*, the causal agent of necrosis [5,6], root rot caused by *Armillaria* spp. [7], leaf spot caused by *Colletotrichum gloeosporioides* [8], and witches' brooms, which is associated with the *peanut stunt virus* [9]. Recently former *Natrassia mangifera,* were re-classified to *Neofusicoccum mangiferae* (Syd. and P. Syd.) Crous, Slippers & A.J.L. Phillips comb. nov. due to extensive taxonomic characterization [10]. The fungus can cause canker and wilt on a wide range of different plant families under humidity and temperatures stress [11]. The fungus was first described as *Hendersonula toruloidea*, a pathogen of deciduous fruit trees in Egypt [12]. Some well-known tree hosts of former *Natrassia mangiferae* are *Eucalyptus camaldulensis*, mulberry, rubber [11], *Acer pseudoplatanus*, *Platanus orientalis*, *Magnolia grandiflora, Eryobotria japonicae*, *Morus alba, Cupressus sempervirens* var. *fastigiata*, *Ulmus procera*, the common fig (*Ficus carica*), and *Ficus benghalensis* [13].

To our knowledge, no specific research has surveyed the phenotypic and molecular characterization of the black locust decline caused by *N. mangiferae*. The main objectives of this study were two-fold. The first objective was to examine the black locust trees for possible causes of decline and death, while the second was to identify the causal agent using routine morphological properties followed by ITS rDNA sequencing.

Materials and Methods

Isolation and cultural characteristics of the pathogens

Wilt and dieback of the black locust were observed during a field survey in Mahalat (33° 54′ N and 50° 27′ E), Markazi province, Iran. Diseased tissues of the aerial parts of the trees showing advanced disease symptoms were collected and disinfected with a sodium hypochlorite solution (1.5% available chlorine) for 3 min. The samples were then rinsed twice with sterile distilled water. Small pieces (0.5–1 cm) of the diseased branches were placed onto a potato dextrose agar (PDA) medium. Pure cultures of fungus were obtained using a mycelia tips culture after incubation at 25°C for three days. Identification of the fungus was made according to the morphological taxonomic system of Moore [14]. Pathogenicity testing was done by wound-inoculation of the black locust branches. The inoculation surfaces were disinfected with 70% ethyl alcohol. A mycelial plug of four-day-old colonies of *N. mangiferae* grown on the PDA was inserted into the stem and branches. The inoculation sites were covered with the removed bark tissues and sealed with Parafilm®. The controls were treated in the same manner with a sterilized agar plug. Three weeks after inoculation, disease symptoms were observed around the inoculation site. Isolation was achieved directly from the lesion to fulfill Koch's postulates.

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DNA extraction and PCR condition

DNA of pure cultures was extracted based on the CTAB protocol [15]. The ITS 4 and ITS 5 primers [16] were used for the amplification of the ITS region (including the 5.8S gene) of the rDNA. PCR amplification was carried out using thermal cycler (Bio-Rad) under the following program: initial preheating at 95°C for 120 s, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 90 s and extension at 72°C for 120 s, with a final extension step at 72°C for 300 s [17]. We used the Maxime PCR PreMix Kit (i-taq) with a total volume of 20 µl by adding 17 µl sterile distilled water, 1 µl (10 pmol/µl) of each primer, and 1 µl (50 ng) of template DNA. The amplified products were separated electrophoretically in 0.8% agarose gels w/v run in TG buffer (3 gr/li Tris-Base MW=121.10, 28.8 gr/li glycine MW=75.07), stained with ethidium bromide (0.08 µg ml^{-1}) for 10 min, visualized under uv light (Gene Snap version 7.09) and the data saved. A DNA standards ladder (100 bp DNA ladder, Fermentaz) was used in each electrophoresis unit. The PCR products were purified by QIAquick PCR purification kit (Qiagen), and then were sequenced using an Automatic Sequencer 3730× (Macrogen Inc., Korea). All amplified sequences were compared with the other sequences of *N. mangiferae* available in the National Center for Biotechnological Information (NCBI) database by using Basic Local Alignment Search Tool (BLAST) [18].

Results

During a survey of urban areas in Iran in 2012, a disease was found to be associated with *R. pseudoacacia*, causing symptoms of wilt and dieback. This fungus infested the stems and branches of the trees with no infection in the root system. Advanced symptoms of the disease produced cankers in the stem and branches without gum exuding. The branches then wilted, followed by wood discoloration from yellowish to black, and the emergence of a sooty layer of fungal arthroconidia.

The characteristics of 18 isolates obtained from affected samples were studied based on morphological characteristics and PCR amplification of the ITS region of the rDNA (ITS4, 5.8S and ITS5). The fungus isolates exhibited no variability in colony characteristics and grew rapidly on a PDA, forming a hyaline-to-gray colony with superficial, immersed, branched, septate mycelia that later became pale to dark. Pycnidia were produced on the PDA three weeks after incubation at 25°C. The pycnidia were superficial, spherical, and the pycniospores were hyaline, 0–2 septate, and an average size of 5.8 µm × 12.3.6 µm.

The arthroconidia were cylindrical, oblong, dark brown to black (Figure 1), and slightly variable in size (average 5.5 µm × 10.5 µm diameter). The optimum growth temperature of the fungus was 28–32°C on the PDA medium. From its cultural and morphological properties and an ITS rDNA analysis, the causal fungus was identified as *Neofusicoccum mangiferae*. These observations were in agreement with the reports of Sutton and Dyko [19] and Crous et al. [10]. The pathogenic disease symptoms were observed as wilting and the decline of the twigs, which finally extended toward the trunk (Figure 2). Lesions were characterized by the production of a yellowish discoloration of the wood surface and the formation of canker on the outer bark surface originating from the point of inoculation (Figure 3). The bark was cracked and readily peeled off, and a sooty layer of fungal spores was produced under the bark (Figure 4). In terms of the pathogenicity on the detached branches, a cottony mass of the fungus had formed in the site inoculated by the fungus plug. There were few differences in pathogenicity among the isolates tested.

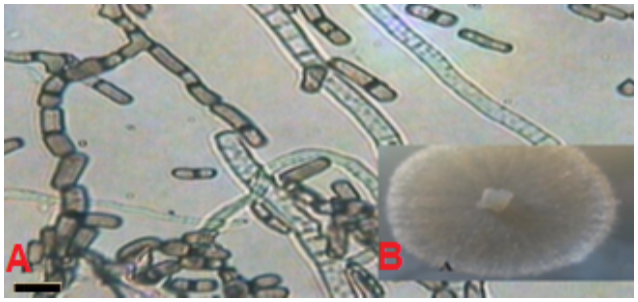

Figure 1: (A) The arthroconidia properties of the fungus, Bar=15 µm. (B) Two-day-old culture of *N. mangiferae* on a PDA medium.

Figure 2: Dead twigs produced after the pathogenicity test in which a mycelia plug was inserted under the bark.

Figure 3: Yellowish discoloration of the wood surface.

The use of the ITS4/ITS5 primers caused the amplification of the expected bands (~560) in the isolates (Figure 5), which was in accordance with the classification of the morphological features employed in this research. BLAST analysis of the sequence showed a high identity correlation with the sequences of the *N. mangiferae* isolates that exist in the National Center for Biotechnology Information (NCBI) databases. The sequence was deposited in GenBank data base under the KP137578 accession number.

Field observations of the disease propagation and past cultural

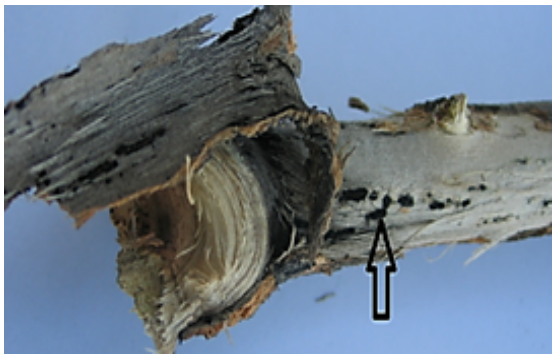

Figure 4: Sooty layer of arthrospores produced under the dead bark.

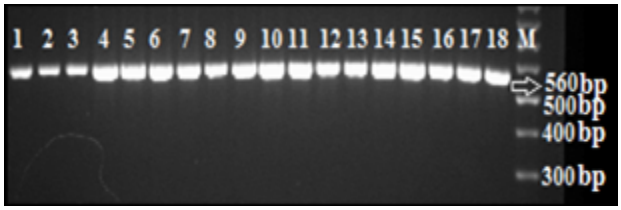

Figure 5: ITS-PCR banding pattern of internal transcribed spacer (ITS) using primer ITS4 and ITS5. Lans1-18: *Neofusicoccum mangiferae* isolated from *Robinia pseudoacacia*, M: molecular marker 100 bp.

histories of different plantings provided evidence that the disease may be spreading rapidly via cultural practices and wind. Hence, there is cause for concern if diseased plants are found among *R. pseudoacacia* and other healthy host plants. Considering the current problem of black locust decline and the importance of developing management programs that are based on the pathogens that occur in the field, morphological and molecular characteristics were used to identify the causal agent that was associated with death of *R. pseodoacacia* in the arid region of Markazi province, Iran.

Neofusicoccum mangiferae is an economically important on different trees and can act as pathogen, primary or secondary that under stress conditions of the host plant can become pathogenic or saprophytic [10,17]. The morphological identification of *N. mangiferae* is based primarily on characteristics of the anamorphs, which are normally found in the field and are easily cultured *in vitro* [17,20]. These criteria include the type of conidial characters such as pigmentation, number of septa, wall thickness and texture, ornamentation and dimensions, culture characteristics, such as colony color [10,20]. This study set out to determine the causal agent of the disease affecting black locust trees in Iran and to study some of the phenotypic, i.e., pathogenic, and molecular properties of the fungus. The threat of this disease in Iran is intensified by the fact that the highly susceptible *R. pseudoacacia* are widely planted throughout the country. We concluded that pruning practices, sunny and hot conditions, and drought stress act as predisposing factors for the development of the disease in the Markazi region. The importance of this result is due to the fact that black locust can act as a powerful repository for the inoculum of *N. mangiferae* for other hosts. It is clear that asexual reproduction is a dominant character for *N. mangiferae* dissemination. Controlling the spread of *N. mangiferae* within and among production sites seems difficult because of the presence of inoculum sources and the broad host range of the pathogen. In many production facilities, plants do not show apparent symptoms before the infection is well established. Indeed, plants that have been treated with protective fungicides may appear healthy until the fungicides efficacy is lost and the pathogen population increases. Overall, adding some minerals to irrigation water, drench irrigation instead of over heading and use of preventive fungicide are recommended for disease management.

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