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ORIGINAL ARTICLE

Neoscytalidium dimidiatum as One of the Fungal Agents Associated with Walnut Decline in Iran

Shima Bagherabadi[1], Doustmorad Zafari[*1], Sajeewa S. N. Maharachchikumbura[2]

[1]*Department of Plant Protection, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran*

[2]*School of Life Science and Technology, University of Electronic Science and Technology China, Chengdu 15 611731, People's Republic of China*

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ABSTRACT

Walnut (*Juglans regia* L.) is one of the main nutritional nut crops in many parts of the world. Iran is one of the most important countries in the world regarding walnut production. In recent years, dieback and decline of some of walnut trees has attracted the attention of growers and plant pathologists in Iran. Although the infestation by the *Cytospora* spp. was accepted as the causal agent of canker, dieback, and decline in walnut trees, the role of the other agents has yet to be well investigated. Field observations were conducted during 2016- 2020 from different provinces in Iran, and black sooty canker was noticed in most declined or declining walnut trees. To determine the causal agent of the observed symptoms, sampling was conducted, and fungal isolates were obtained from symptomatic tissues of walnut trees. The fungus consistently isolated was identified as *Neoscytalidium dimidiatum* based on morphological and molecular characteristics of the ITS and *tef1-α* regions. *Neoscytalidium dimidiatum* was shown to be the cause of the symptoms by pathogenicity testing. The current findings show that *N. dimidiatum* is a serious and devastating disease in Iran, causing walnut tree decline.

Introduction

Persian walnut (*Juglans regia* L.), one of the major nut crops in the family *Juglandaceae*, is a valuable tree that is found in several parts of the world (Mousivand *et al*., 2013; Sarikhani *et al.*, 2021; Thapa *et al.*, 2021). It is the second-most important edible nut crop grown in Iran, the third-largest walnut producer globally (Hassani *et al*., 2020). Walnut is a worth crop that is generally consumed (Akca and Sahin, 2022). Not only nuts but also all components of walnuts are being used in both cosmetic and pharmaceutical industries (Vahdati *et al*., 2018). Being sessile, plants are often exposed to the soil-born pathogens of contaminated soils or other sources of pathogens (Aoko *et al.*, 2021). The walnut trees are infected by a wide range of fungal and bacterial pathogens (Khodadadi *et al.*, 2016; Farr and Rossman, 2021). Among them, branch and trunk canker diseases have become prevalent on nut crops (Gramaje *et al*., 2016). Species of *Cytospora* are one of the most important fungal pathogens associated with walnut canker disease in the world (Fan *et al*., 2015). *Cytospora* canker caused by the fungus *Cytospora* spp. is one of the most common diseases of walnut trees in many parts of Iran. The disease typically attacks walnut branches and leads to large areas of dieback (Abbasi *et al*., 2013). The decline is a multidimensional and complex phenomenon and is caused by a broad range of plant pathogens. The severity of symptoms, when the disease occurs, is driven by several factors that take place simultaneously or frequently. Although infestation by the *Cytospora* spp was accepted as

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a fungal agent of dieback and canker in walnut trees, the role of the other agents has yet to be well investigated. Some members of the family *Botryosphaeriaceae* are well-known pathogens causing dieback, canker, fruit rot and associated with the decline of several woody hosts (Mayorquin *et al*., 2016; Lou *et al*., 2019; Berraf-Tebbal, 2020), including walnut (Chen *et al*., 2014). *Neoscytalidium dimidiatum* is an opportunistic pathogen of different hosts causing several kinds of diseases (Padin *et al*., 2005). In Iran, *N. dimidiatum* was reported to cause cankers and wilt on a wide range of hosts. In recent years, a fungal disease called sooty canker caused by *N. dimidiatum* has been identified as associated with the decline on various hosts including *citrus* and *Ficus* spp. (Polizzi *et al*., 2009; Al-Bedak *et al*., 2017). A serious decline and dieback of plum trees were reported to be caused by *N. dimidiatum* in Tunisia (Hajlaoui *et al*., 2018). *N. dimidiatum* caused canker and dieback of eucalyptus in Arizona (Matheron and Sigler 1993), dieback on mango and common fig in Australia (Ray *et al*., 2010). The fungus was also associated with canker disease in Egyptian *Ficus* trees (Al-Bedak *et al*., 2017). In Turkey, *N. dimidiatum* had previously been reported as a new blight of tomato (Türkölmez *et al*., 2019). In a study conducted by Dervi *et al*., (2019), *N. dimidiatum* causing canker, shoot Blight, and root rot of Pistachio. In Iran, several reports of this fungus were published on different hosts. Branch wilt, decline and death of citrus reported by *N. dimidiatum* (Alizadeh *et al*., 2000). This species was recorded as a causal agent of dieback and death on olive trees (Sanei and Razavi 2012). *N. dimidiatum* caused the canker disease of apple trees in Iran (Nourian *et al*., 2013). *N. dimidiatum* was also reported to cause wood lesions, dieback, canker, and decline on willow and poplar trees (Hashemi and Mohammadi, 2016), *Calligonum amoenum* (Nazmadini *et al*., 2018), and date palm (Rahiminiya *et al*., 2018). Mirtalebi *et al*., (2019) was reported *N. dimidiatum* as a causal agent of fruit rot on melon and *Salix* sp. In a study conducted by Aminaee and Ershad (1993), *N. dimidiatum* was recorded to cause canker and wilt on pomegranate. *N. dimidiatum* has been isolated from citrus (Heydarian and Minasiyan 1995), *Ficus religiosa,* and *Psidium guajava* (Mirzaee *et al*., 2002), and *Pistachia vera* and *Punica granatum* (Ghelichi *et al*., 2012). First occurrence of canker and dieback on *Avicennia marina* and *Rhizophora mucronata* caused by *N. dimidiatum* was reported by Goudarzi and Moslehi (2019).

In recent years, the incidence of canker, dieback, and decline of walnut trees has increased and caused economic losses in Iran. During a survey of declining and declined walnut trees, black sooty canker symptoms were observed on branches and trunks of walnut trees in different provinces of Iran. Since these symptoms were observed in most declining or declined trees, it seems that the causative agent of these symptoms could be an important role in the decline of walnut trees. The main objectives of this study were to determine the causative agent of black sooty canker based on morphological, molecular, and pathogenicity studies.

Materials and Methods

Sampling, isolation, and morphological characterization

Superficial bark lesions characterized diseased walnut trees with a black powder underneath the bark, branch wilt, trunk and branch cankers, dieback, gradual decline, and sometimes complete death of trees. Peeling off the thin outer layer of the bark and exposing the black sooty mass of spores was the main characteristic feature of this disease (Fig. 1 b). Field surveys were conducted from important walnut-growing provinces in Iran from 2016 to 2020 (Table 1). The collected samples were placed in paper bags, labeled, transported to the laboratory, and stored at 4°C for further studies. Fungal isolates were obtained using two methods as follow: In the first method, the black sooty layer underneath the bark tissues was peeled off from the trunk and branch samples using a sterile needle, and transferred to potato dextrose agar (PDA; 200g potatoes, 20g dextrose, 15g agar, 1 l water). In the second method, small pieces were cut from the boundary between diseased and healthy tissues, surface-sterilized with 1% (w/v) sodium hypochlorite for 2 min, rinsed three times with sterile water, dried on sterile filter paper, and then transferred to PDA plates. The culture plates were incubated at 25ºC in the darkness. Fungal isolates were transferred to fresh PDA

plates three to five days after incubation, and pure cultures were produced using the single spore isolation technique (Ho and Ko, 1997). All single conidial isolates were examined morphologically using a BX53 microscope (Olympus, Tokyo, Japan). Colony morphology, as well as size and shape of conidia, were recorded after seven days of incubation on PDA at 25°C in the darkness. The dimensions of 30 randomly selected conidia were measured. As the cultural and morphological traits of isolates were very similar to each other, five isolates (each province, one isolate) were selected as representative isolates for molecular analysis and pathogenicity tests, and a culture of representative isolates (SB226) was deposited in the Iranian Research Institute of Plant Protection Culture Collection (Table 1).

Fig. 1. (a-c) Natural symptoms of black sooty canker caused by *Neoscytalidium dimidiatum* on walnut trees. (d-e) Artificial symptoms after inoculation with *N.dimidiatum* in laboratory conditions. (f) Control shoot without any symptoms.

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from mycelium grown on PDB (Potato Dextrose Broth) as described by Moller et al. (1992). The internal transcribed spacer (ITS) region of rDNA was amplified using primers ITS1 and ITS4 (White et al. 1990), and additional translation elongation factor 1-alpha (*tef1-α*) gene region was amplified using primers EF1-728F/EF1986R to improve species delineation within the genus *Neoscytalidium*. The PCR reaction was performed in a TC-512 thermal cycler (Techne, Germany) in a total volume of 25µl. The 25µl reaction volume contained 10 ng of genomic DNA, 1µM of each primer, 0.2 mM of dNTPs (CinnaGen, Iran), 2.5 µL 10X PCR buffer, 2.5 mM $MgCl_2$, and 1 U Taq DNA polymerase (CinnaGen, Iran). PCR reaction was set as follows: initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 56°C (for ITS) and 52°C (for *tef1-α*) for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were purified and sequenced by Bioneer Corporation (South Korea). Sequences generated from different primers (ITS1/ITS4 and EF1-728F/EF1986R) were analyzed with other sequences obtained from GenBank. Alignments for each locus were generated with BioEdit v.7.0.5.2 (Hall, 1999), and manual adjustments of alignments were made when necessary. Homology searches were performed using the BLAST program of the National Center for Biotechnology Information (NCBI) for the preliminary identification of isolates used in the analysis and additional reference sequences were obtained from GenBank. TEF and ITS sequences of each species (including type specimen sequences downloaded from GenBank) were concatenated and aligned in the CLUSTAL-X v. 1.81 (Thompson *et al*., 1997). Maximum

Likelihood (ML) analysis was performed on the combined data set of ITS ribosomal DNA and *tef1-α* gene region sequences of representative isolates from *Juglans regia*, Ex-type isolate of *N. dimidiatum*, and sequence of species closely related to *N.dimidiatum* from GenBank in MEGA 7 (Kumar *et al*., 2018) with the Kimura-2-parameter distance model (Kimura, 1980). The robustness of the analyses was evaluated by 1000 bootstrap support (Felsenstein, 1985). The tree was rooted to *Neofusicoccum mangiferae* (CMW7024) (Kee *et al*., 2017; Monteles *et al*., 2020). The nucleotide sequences reported in this paper were deposited in GenBank (Table 1).

Table 1. Details of species and sequences included in the phylogenetic analyses

Species	Strain/Culture Collection Number	Host	Location	GenBank NO.	
				ITS	EF-1α
Neoscytalidium dimidiatum	**SB 1**	***Juglans regia***	**Kerman, Iran**	**MZ675434**	**MZ683486**
N. dimidiatum	**SB 54**	***Juglans regia***	**Kermanshah, Iran**	**MZ675435**	**MZ683487**
N. dimidiatum	**SB226 (IRAN 4520C)**	***Juglans regia***	**Hamedan, Iran**	**MZ675436**	**MZ683488**
N. dimidiatum	**SB 303**	***Juglans regia***	**Kurdistan, Iran**	**MZ675437**	**MZ683489**
N. dimidiatum	**SB 613**	***Juglans regia***	**Esfahan, Iran**	**MZ675440**	**MZ683492**
N. dimidiatum	CBS 499.66 [T]	*Mangifera indica*	Mali	KF531820	KF531798
N. novaehollandiae	CBS 122071[T]	*Crotalaria medicaginea*	Australia	EF585540	EF585580
Neofusicoccum mangiferae	CMW7024	*Mangifera indica*	Australia	AY615185	DQ093221

[1] T: The CBS accession number indicates ex-type strains.

[2] Details of obtained isolates in this study are in **bold**.

Pathogenicity test

To fulfill Koch's postulates, pathogenicity tests were conducted on healthy excised shoots in laboratory conditions. In total, 18 healthy shoots of walnut trees were used in the pathogenicity tests. To confirm the results, each representative isolate was inoculated in triplicates, and the experiment was repeated twice). The leaves were removed, and healthy shoots were surface sterilized with 70% ethanol. Inoculations were made after removing a 5-mm bark disc using a sterile 5 mm diameter metal cork borer. A mycelial plug (5 mm diameter) obtained from the margin of a seven-day-old culture on PDA was inserted into the wound. The wounds were sealed with vaseline and parafilm to prevent contamination and desiccation. For the control shoots, plugs of clean PDA were placed in the wounds. Inoculated shoots were placed in a plastic container containing moistened filter papers to keep the relative humidity high and were kept in laboratory conditions at 25°C with natural daylight. Symptoms and disease development were monitored on inoculated shoots regularly. To test Koch's postulates, re-isolation was carried out using the same approach as for preliminary isolation and morphological comparisons were made with the initial isolates.

Results

Disease symptoms and morphological characterization

Characteristic disease symptoms were a black sooty layer of conidia under bark, dieback, and decline of walnut trees. In some cases, the whole of the tree was dried, and in other cases, a part of the tree that had disease symptoms was only dried (Fig1a & c). In total, 64 isolates were obtained from declining or declined walnut trees in this study. All isolates were very fast-growing, and fungal mycelia grew out after two days. Colony color on PDA initially was white appearing powdery in the center, changed to an olive-green to greyish color, and finally turned dark-gray to black (Fig. 2a). Colonies produced mycelia that disarticulated into 0- to 1-septate, cylindrical to round, brown arthroconidia were arranged in arthric chains or singly. The arthroconidia were different in morphology and coloration. The arthroconidia were 5.5 – 9

× 3.5–5 µm in size (Fig. 2b, c). The cultural and morphological characteristics of the isolates were in agreement with the description for *N. dimidiatum* (Penz.) Crous & Slippers (Crous *et al*. 2006).

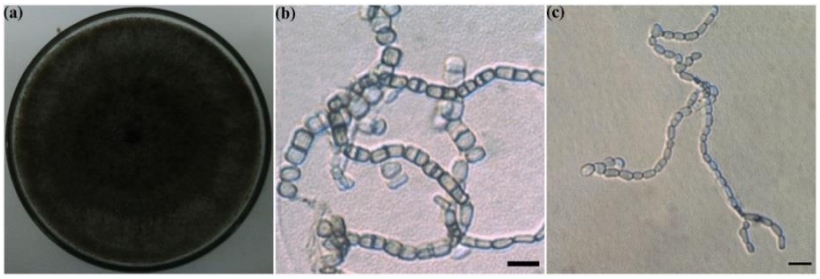

Fig. 2. *Neoscytalidium dimidiatim* SB 226 after 7 days on PDA (a) Surface of colony. (b-c) The variable shape of arthroconidia in a chain. Scalr bars = 10 µm

Phylogenetic analysis

Based on the multigene phylogenetic analysis, the isolates were identified as *N. dimidiatum* per morphological identification. Maximum Likelihood (ML) analysis showed that one major clade (72% bootstrap support) was formed from the combined dataset. This clade corresponds to *N. dimidiatum*, which includes isolates obtained from walnut trees in this study and reference isolate of *N. dimidiatum* (CBS 499.66) with high support (Fig. 3).

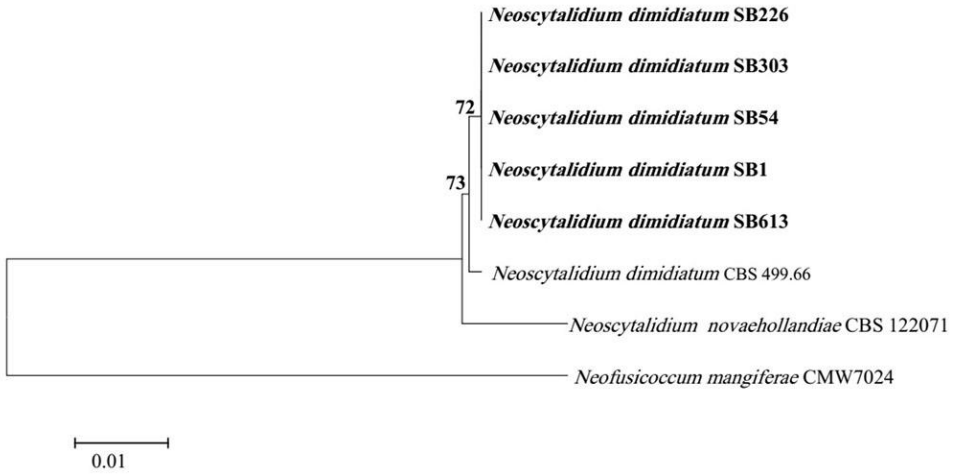

Fig. 3 Phylogenetic tree of Maximum Liklihood analysis of *Neoscytalidium dimidiatum* isolates from this study (indicated in bold) with references isolates of *Neoscytalidium* spp. based on the internal transcribed spacer region of rDNA (ITS) and translation elongation factor 1-alpha (tef1-α) gene region. *Neofusicoccum mangiferae* (CMW7024) is selected as outgroup. Bootstrap values (>70%) are shown as percentages of 1000 replicates at the node.

Pathogenicity test

The first visible disease symptoms appeared 7 days after inoculation. Peeling away the bark of the inoculated shoots exposed the presence of black spore masses underneath the bark, while no symptoms appeared on the control shoots. *Neoscytalidium dimidiatum* was consistently re-isolated from artificially infected shoots; therefore, Koch's postulates fulfilled (Fig. 1d, f).

Discussion

It is well-known that *Cytospora* species are destructive canker and dieback pathogens of woody hosts in natural and agroecosystems worldwide (Lawrence *et al*., 2018). The decline disease mainly influences walnut branches, which leads to large areas of dieback, thus inflicting economic losses, but *Cytospora* cannot be the only cause of this phenomenon in Iran.

This study represents the first detailed assessment of the morphological and molecular identification along with pathogenicity of black sooty canker agent associated with walnut dieback and decline in the major walnut-growing provinces of Iran. In total, 64 isolates were obtained from declining or declined walnut trees in this study. Some walnut trees were thoroughly dried up or declined, but only one side or just a few branches were damaged for some other walnut trees. Dieback and decline of walnut trees is an ongoing problem of walnut orchards. In recent years, the incidence of dieback and decline has increased in Iran. The common incidence of black sooty canker on declining and declined trees suggests that *N. dimidiatum* is associated with the decline of walnut trees in Iran. *Botryosphaeriaceae* are important pathogens on a variety of woody hosts, including walnut, a major crop in Iran. As a member of this family, this species is an opportunistic pathogen of different hosts when the affected plants are under stress conditions worldwide (Padin *et al*., 2005). Since this species has a wide host range and distribution as an opportunistic pathogen leads to different diseases. It is often reported as one of the agents associated with declining trees, trees with symptoms including dieback and wilting, and was isolated from fruit and shade trees around the world. The main source of inoculation and resistant form of this fungus are arthospores, which are spread by wind and rain also weakened trees are more susceptible to this fungus. Based on previous findings and the results of this study, it was hypothesized that *N. dimidiatum* is an opportunistic plant pathogen that can also be involved in the decline of walnut trees in Iran when biotic or abiotic agents weaken the tree. This species previously caused canker, shoot blight, and fruit rot on walnut trees in California (Nouri *et al*., 2018) and black canker and root rot of walnut in Turkey (Derviş *et al*., 2019). The commercial loss owing to the pathogen could be quite significant because it causes the death of young trees and decreases the economic life of old trees. Based on the morphological and molecular characteristics of ITS and *tef1-α* regions, and necrosis and bark peeling produced on the inoculated plants, *N. dimidiatum* was identified as the causal agent of sooty cankers in *J. regia* trees in Iran. The present results also revealed that *N. dimidiatum* is one of the most destructive pathogens associated with the decline of walnut trees in Iran, and it could have a significant potential for limiting walnut production. Since Iran is an important origin and cultivation center of walnut in the world with a rich walnut production (Vahadati *et al*., 2018) therefore; management of the disease is urgently needed for the walnut economy of Iran.

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