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Morphological and molecular characterization of *Neoscytalidium novaehollandiae*, the cause of *Quercus brantii* dieback in Iran

Mohammad SABERNASAB, Samad JAMALI*, Alireza MAREFAT, Saeed ABBASI

Department of Plant Protection, College of Agriculture, Razi University, Kermanshah, Iran
*Corresponding author: jamali454@yahoo.com

Summary. During the period from 2013 to 2015 a tree health survey was conducted in Persian oak (*Quercus brantii* Lindl.) forests throughout Kermanshah province in Western Iran. Oak trees showing dieback of branches and stems were sampled, and fungal colonies resembling those of *Neoscytalidium* sp. were obtained from diseased tissues. Based on morphology and phylogeny of DNA sequence data for the internal transcribed spacer (ITS) rDNA, nuclear ribosomal large subunit (LSU) and translation elongation factor 1-alpha (TEF 1α) gene regions, all isolates were identified as *Neoscytalidium novaehollandiae*. Pathogenicity tests were carried out on 2-year-old, potted *Q. brantii* plants and on detached branches under partially controlled conditions. Pathogenicity tests showed that all the isolates were able to infect, and cause disease symptoms on, inoculated branches and plants. This is the first report of *N. novaehollandiae* as the causal agent of *Q. brantii* dieback.

Keywords. Brant's oak, Persian oak, emerging fungal pathogens, Kermanshah forests.

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INTRODUCTION

Zagros oak forests are the largest forest type in Iran, covering ca. 40% of the country's forest area (Sagheb-Talebi *et al*., 2004). The genus *Quercus* L. (*Fagaceae*) is one of the most diversified groups of temperate trees, with approx. 500 species distributed worldwide (Mehrnia *et al*., 2012). Kermanshah oak forests in West Iran are currently affected by serious oak dieback symptoms, including extensive leaf yellowing and dieback of branches and stems. Recent studies showed that numerous pathogenic fungi and oomycetes are involved in the dieback, including *Biscogniauxia mediterranea*, *Fusicoccum quercus*, *Ophiostoma querci*, *Diplodia mutila, Apiognomonia quercina, Stereum rugosum*, *Pezicula cinnamomea*, *Ceratocystis fagacearum*, *Dothiorella iberica*, and *Phytophthora ramorum* (Simonin *et al*., 1994; Grünwald *et al*., 2012; Mirabolfathy, 2013; Lynch *et al*., 2013).

The genus *Neoscytalidium* (Botryosphaeriaceae) was recently established by Crous *et al*. (2006). *Neoscytalidium dimidiatum* (Penz.) Crous and Slip-

pers, has a broad host range and wide distribution (Ray *et al*., 2010), and has often been reported to be associated with trees showing symptoms of decline, such as wilting and dieback. The fungus has been isolated from deciduous fruit trees and shade trees in Egypt, South Tunisia, California, Jamaica, Iraq, Niger, West Africa, India, China and Europe (Nattrass, 1933; Calavan and Wallace, 1954; Sommer, 1955; Meredith, 1963; Natour and El-Haider, 1967; Giha, 1975; Reckhans and Adamou, 1987; Cao and Wang, 1989; Granata and Sidoti, 1991; Harsh and Tiwari, 1992; Matheron and Sigler, 1993; Elliott *et al*., 1997; Msikita *et al*., 1997; Tsahouridou and Thanassoulopoulos, 2000; Elshafie and Ba-Omar, 2001; Namsi *et al*., 2010). Pavlic *et al*. (2008) described a new species isolated from asymptomatic and dying branches of *Acacia synchronica*, *Adansonia gibbosa*, *Crotalaria medicaginea* and *Grevillea agrifolia*, and named it as *Neoscytalidium novaehollandiae*. This fungus has since been reported as a pathogen of *Mangifera indica* in Australia (Ray *et al*., 2010), and was isolated from bark beetle galleries in *Ulmus densa* in China (Zhu and Liu, 2012). In Iran, *N. dimidiatum* has been isolated from *Ficus religiosa*, *Psidium guajava*, *Pistachia vera* and *Punica granatum* (Aminaee and Ershad, 1993; Ghelichi *et al*., 2012).

Despite the importance attributed to *Neoscytalidium* species as disease agents of fruit and forest trees, no study has been carried out to investigate a possible association between *N. novaehollandiae* infections and the extensive oak dieback that has been recently observed in Kermanshah province in Western Iran. The aim of the present study was to verify this putative association by isolating and identifying *Neoscytalidium* species from *Quercus brantii* individuals showing dieback symtoms in Iran, and to clarify the possible involvement of these fungi in the disease by means of artificial inoculations in controlled conditions.

MATERIALS AND METHODS

Sampling and morphological characterization of fungi

During the period from 2013 to 2015, a tree health survey was conducted in *Q. brantii* forests throughout Kermanshah province in Western Iran. Samples were collected from oak trees exhibiting symptoms of yellowing of the leaves and dieback of branches, and these were brought to the plant pathology laboratory in the Department of Plant Protection of Razi University, Kermanshah for further examination. Wood pieces were surface sterilized by 0.5% sodium hypochlorite for 1–3 min, rinsed with sterile distilled water, blotted dry with sterile paper towels and plated onto potato dextrose agar (PDA) and malt extract agar (MEA) amended with chloramphenicole (25 μg mL^{-1}). Micromorphological features of the fungal mycelium were studied using a light microscope (Olympus model BX-51), and images were captured with a camera (Canon Powershot model SX10). Fifty measurements of the observed fungal structures, including arthrospores, muriform conidia and pycnidiospores, were made using the BioloMICS software (Robert *et al*. 2011).

Molecular phylogenetic identification of fungi

The DNA was extracted from 5-d-old mycelium from seven isolates cultivated on PDA, using the method described by Gardes *et al*. (1991). The ITS-1 and ITS-4 primer pairs (White *et al*., 1990) were used to amplify the ITS1+5.8S+ITS2 of the ribosomal RNA. The EF1F and EF2R primer pair (Burgess *et al*., 2005) was used to amplify the portion of the translation elongation factor 1-alpha (EF1) gene. The LROR and LR5 primer pair (Slippers *et al.,* 2013) was used to amplify the nuclear ribosomal large subunit (LSU) gene region. All PCR reactions were conducted in 25 μL containing 20 ng genomic DNA, 1 μM of each primer, 100 μM of each dNTP, 0.5 U Taq DNA polymerase (CinnaGen, Iran), 1.5 mM of MgCl2, 2.5 μL of 10 × PCR buffer (CinnaGen, Iran), and 14.5 μL H_2O. The PCR reactions were performed in a T-Personal thermocycler (Biometra). PCR conditions were as follows: an initial denaturation step of 5 min at 95°C, 35 cycles each at 95°C for 60 s, annealing at 55°C for 80 s, elongation at 72°C for 90 s, followed by a final elongation step of 10 min at 72°C. The amplification products were visualized by electrophoresis in a 1% TBE-agarose gel. After sequencing (Tech Dragon,), the nucleotide sequences were edited using BioEdit Sequence Alignment Editor v. 7.2.5 software (Hall, 1999), and a similarity search in the GenBank sequence database was performed using BLAST service in NCBI (http://blast.ncbi.nlm.nih.gov). All sequences were deposited in GenBank. For phylogenetic analysis, ITS, LSU and EF1 data sets from this study were combined with sequences of *N. novaehollandiae* downloaded from GenBank (Table 1). *Lecanosticta acicola* and *Spencermartinsia viticola* were used as an outgroup (Slippers *et al*., 2013). DNA sequences were aligned with ClustalW (http://www.clustal.org/download, Thompson *et al.* 1994), and manually edited with BioEdit v. 7.2.5. Phylogenetic analyses were performed with MEGA5 software (http://megasoftware.net/) using neighbor-joining and maximum likelihood (ML) methods (Tamura *et al*., 2011). The best fit nucleotide substitution model (Tamura 3-parameter) was based on the

Table 1. Isolates of fungi used in this study, their locations and their NCBI GenBank. Sequence accession numbers for the Large Subunit of the nuclear ribosomal RNA (LSU), Internal Transcribed Spacer (ITS) and Translation elongation factor 1-alpha (EF-1) gene portions.

Species	Isolate ID	Location	GenBank Accession No.		
			LSU	ITS	EF1
Neoscytalidium novaehollandiae	CMW 26170	South Africa	KF766374	-	-
N. novaehollandiae	CBS 122071	Australia	-	KF766207	EF585580
N. novaehollandiae	NeNo1	Iran	MH899579	KY499712	MF662595
N. novaehollandiae	NeNo2	Iran	MH899580	KY499713	MF662596
N. novaehollandiae	NeNo3	Iran	MH899581	MH883623	MH885094
N. novaehollandiae	NeNo4	Iran	MH899582	MH883624	MH885095
N. novaehollandiae	NeNo5	Iran	MH899583	MH883625	MH885096
N. novaehollandiae	NeNo6	Iran	-	MH883626	MH885097
N. novaehollandiae	NeNo7	Iran	-	MH883627	MH885098
N. novaehollandiae	WAC12688	Australia	NG059496	EF585543	EF585575
N. dimidiatum	IP127881	Netherlands	DQ377925	AY819727	EU144063
N. dimidiatum	NDFB003	Mexico	MF508740	EF585537	EF585577
N. dimidiatum	CBS:125695	Netherlands	KX464535	GU172385	GU172417
N. dimidiatum	CBS:125616	Netherlands	KX464534	GU172389	GU172421
N. dimidiatum	CBS 312.90	Netherlands	DQ377924	GU172383	GU172415
Barriopsis fusca	CBS 174.26	Netherlands	KF766317	KF766149	KF766395
B. iraniana	IRAN 1448C	Iran	KF766318	KF766150	FJ919652
Botryosphaeria agaves	MFLUCC 10-0051	Thailand	JX646807	JX646790	JX646855
B. corticis	CBS 119047	Netherlands	EU673244	DQ299245	EU017539
B. dothidea	CMW 8000	South Africa	KF766319	KF766151	AY236898
Cophinforma atrovirens	MFLUCC 11-0425	Thailand	JX646817	JX646800	JX646865
C. atrovirens	MFLUCC 0655-11	Thailand	JX646818	JX646801	JX646866
Dichomera saubinetii	CBS 990-70	Netherlands	DQ377888	KF766153	KF766396
Diplodia africana	CBS 120835	Netherlands	KF766322	KF766155	KF766397
D. allocellula	CMW:36468	South Africa	JQ239410	JQ239397	JQ239384
D. allocellula	CMW:36469	South Africa	JQ239411	JQ239398	JQ239385
Dothiorella brevicollis	CMW:36463	South Africa	JQ239416	JQ239403	JQ239390
D. brevicollis	CMW:36464	South Africa	JQ239417	JQ239404	JQ239391
Endomelanconiopsis endophytica	CBS 120397	Netherlands	EU683629	KF766164	EU683637
E. microspora	CBS 353.97	Netherlands	KF766330	KF766165	EU683636
Kellermania anomala	CBS 132218	Netherlands	KF766343	KF766173	KF766404
K. confusa	CBS 131723	Netherlands	KF766344	KF766174	KF766405
Lasiodiplodia crassispora	CBS 118741	Netherlands	DQ377901	DQ103550	EU673303
L. gonubiensis	CMW 14077	South Africa	KF766361	KF766191	DQ458877
L. parva	CBS 456.78	Netherlands	KF766362	KF766192	EF622063
L. pseudotheobromae	CBS 116459	Netherlands	EU673256	KF766193	EF622057
Macrophomina phaseolina	CBS 22733	Netherlands	KF766364	KF766195	KF766422
Neofusicoccum eucalypticola	CMW 6539	South Africa	KF766368	KF766201	AY615133
N. luteum	CMW 10309	South Africa	KF766369	KF766202	KF766424
N. parvum	CMW 9081	South Africa	KF766371	KF766204	KF766426
Phaeobotryosphaeria eucalypti	MFLUCC 11-0579	Thailand	JX646819	JX646802	JX646867
P. porosa	CBS 110496	Netherlands	KF766375	KF766210	EU673130
Pseudofusicoccum adansoniae	CMW 26147	South Africa	KF766386	KF766220	EF585571
P. ardesiacum	CMW 26159	South Africa	KF766387	KF766221	EU144075
Spencermartinsia viticola	CBS 117006	Netherlands	KF766392	KF766228	AY905559

bayesian information criterion (BIC) and was implemented in MEGA 5. The confidence values of phylogenetic trees were assessed by calculating 1,000 bootstrap re-samplings.

Growth studies

All fungus isolates obtained from oak trees from throughout Kermanshah province were transferred to PDA plates and incubated at different temperatures at 5°C intervals from 10 to 45°C. Three plates were used for each isolate and temperature combination. Diameters of individual colonies were measured after 1 week of incubation.

Laboratory pathogenicity test

All isolates (n = 14) were used for laboratory pathogenicity testing on detached branches, under partially controlled conditions. The middle parts of healthy oak branches (each 20 cm long) were surface sterilized with 75% ethyl alcohol. A superficial wound was made in each branch by plunging a 10 mm diam. cork borer into the bark to a depth of 0.5 cm, and a mycelium plug obtained from the margin of a growing fungal colony was placed in the wound and wrapped with Parafilm. Inoculated branches were stored in sterilized glass jars each containing a moist sponge, and were then incubated on a 12 h : 12 h light:dark cycle at 25°C for 25 d until the appearance of the disease symptoms (Banihashemi and Javadi, 2009).

Greenhouse pathogenicity test

Pathogenicity of the isolates was also tested in a greenhouse under partially controlled conditions (natural day/night length, 25–27°C, 60–70% RH). Stems (approx. 15 mm diam., and 25-30 cm length) from 2-year-old seedlings (three plants per fungal isolate) were used. The stems were each inoculated ten cm above soil line using a 5 mm diam. cork borer to expose the cambium. A plug of mycelium (5 mm diam.) was placed into the wound, with the mycelium facing the cambium, and the inoculation point was sealed with Parafilm to reduce desiccation. The length of lesions that formed under the stem bark and on the cambium were measured after 6 weeks. Surface sterilized stem pieces were then taken from necrotic tissues and were plated on PDA, and if *N. novaehollandiae* was re-isolated, Koch's postulates were fulfilled.

Host range study

Detached branches of seven woody plant species sampled from the studied oak forest, including *Ficus carica*, *Acer monspessulanum*, *Crataegus aronia*, *Amygdalus scoparia*, *Cornus mas*, *Pistacia atlantica* and *Pinus eldarica,* were inoculated with three representative fungal isolates under laboratory conditions, using the method described above. Symptoms developing on the branches were assessed 25 d after inoculation.

RESULTS AND DISCUSSION

Fourteen isolates resembling *Neoscytalidium* were recovered from diseased oak trees displaying dieback symptoms, collected in oak forests throughout Kermanshah province in Western Iran. Affected oak trees showed various symptoms including; yellowing of the leaves, dieback of branches and stems, reddish-brown cankers on branches, and internal dark brown vascular necrosis in cross sections of the branches and stems (Figure 1). *Neoscytalidium*-like colonies were consistently isolated from symptomatic tissues on PDA and MEA. Fungal colonies in these cultures were dark blackish, and the hyphae were medium to dark brown and smooth. Arthroconidia were medium to dark brown, smooth, mostly non-septate but occasionally with one dark transverse septum, cylindrical, spherical to subspherical and measured 4–10 × 3–6 µm (Figure 2D). Dictyospores were spherical to subspherical, measuring 6.7–10.3 × 4.8–9.9 µm (Figure 2E).

All the *Neoscytalidium*-like isolates that were sequenced showed 100% sequence homology with *N. novaehollandiae* (GenBank KF766207, Slippers *et al*., 2013) at all three sequenced regions, i.e. ITS (Genbank accession nos KY499712, KY499713, MH883623, MH883624, MH883625, MH883626, MH883627), EF1 (accession nos MF662595, MF662596, MH885094, MH885095, MH885096, MH885097, MH885098) and LSU (accession nos MH899579 to MH899583). In phylogeny trees based on ITS, LSU and EF1 sequences, isolates from Iran clustered to a distinct monophyletic clade together with *N. novaehollandiae* isolates described by other authors (Figures 3 and 4).

Based on morphology and phylogenetic analyses, all isolates were identified as *N. novaehollandiae*. Pavlic *et al*. (2008) showed that *N. novaehollandiae* produced muriform, Dichomera-like conidia that distinguish this species from known *Neoscytalidium* species. The ITS regions of the genomic ribosomal RNA gene and part of the tranlation elongation factor 1-alpha gene were previ-

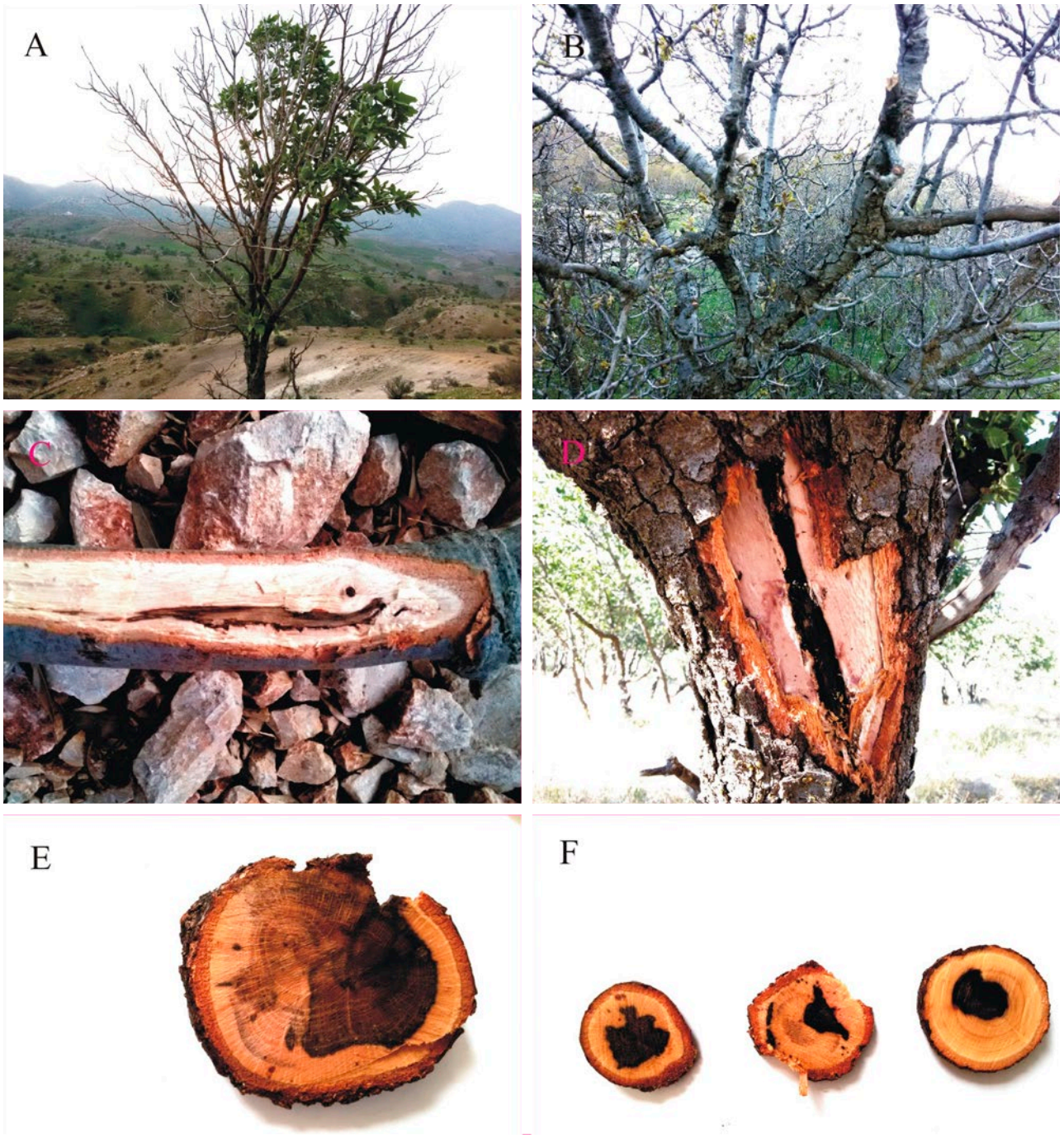

Figure 1. Symptoms of dieback and decline (A and B), canker (C and D, longitudinal sections), and watery necrosis and back staining (E and F cross sections), caused by *Neoscytalidium novaehollandiae* on Persian oak trees in Iran.

ously successfully applied for discriminating *Neoscytalidium* species (Burgess *et al*. 2005; Ray *et al*. 2010). An inferred phylogeny based on ITS, LSU and EF1 sequences clustered the isolates in a distinct monophyletic group consisting of sequences of *N. novaehollandiae* generated in previous studies (Figures 3 and 4). The phylogenetic trees inferred by both neighbor-joining (not shown) and maximum likelihood methods showed very similar topology, although there were minor differences in the bootstrapping percentages.

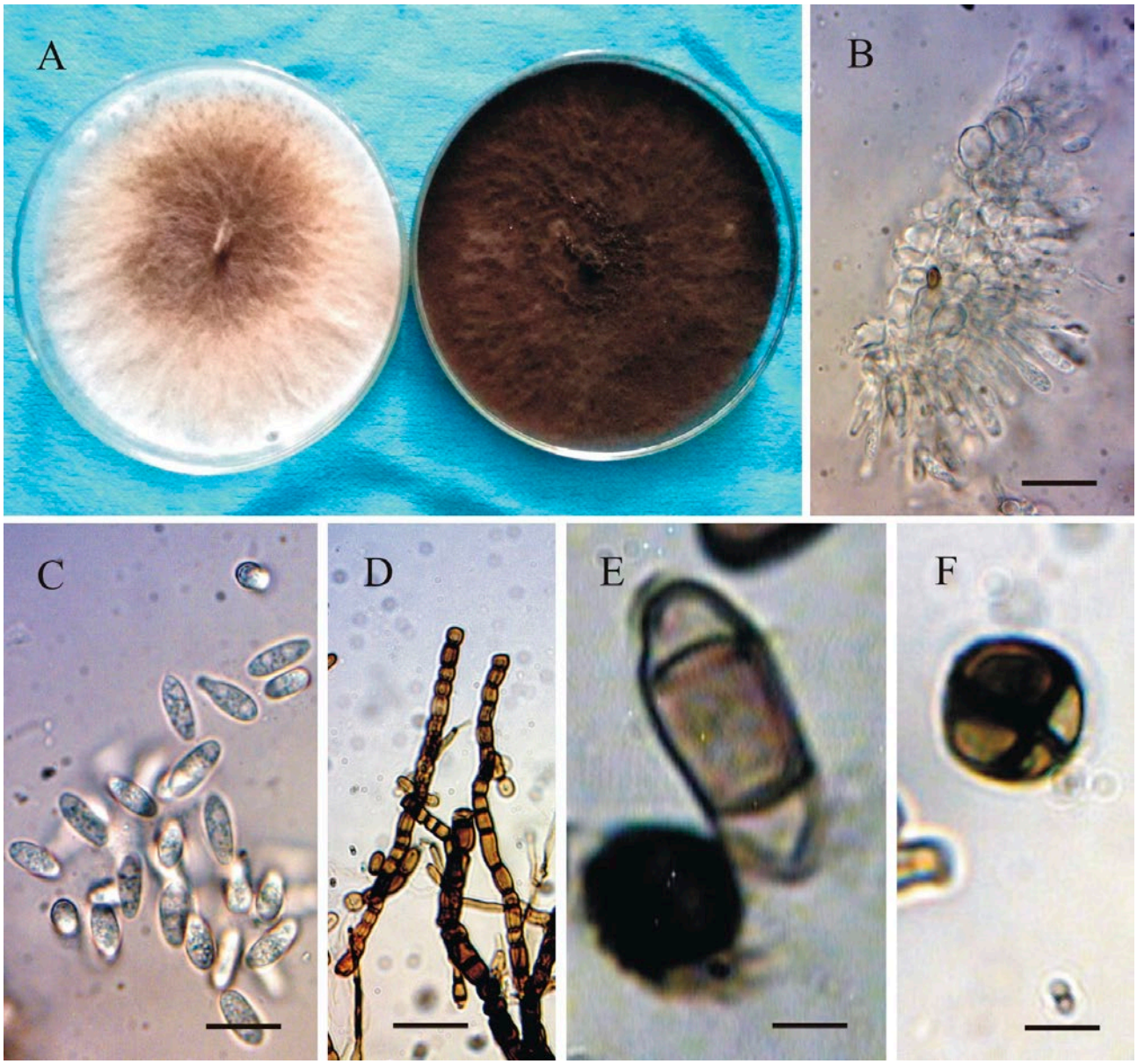

Figure 2. Morphological features of *Neoscytalidium novaehollandiae* isolates from diseased Persian oak trees: A,1-week-old colony grown on PDA; B, conidiogenous cells with developing conidia; C, immature conidia; D, arthrospores; E, mature conidium; and F, muriform conidia. (Bars = 5 µm in and 17 µm in B. C, D and F).

Pathogenicity tests revealed that all 14 isolates of *Neoscytalidium* from this study were pathogenic to *Q. brantii* seedlings. Fourty five days after inoculation, cankers extending both upward and downward from the points of inoculation were evident on the stems of all the inoculated seedlings (Figure 5). Inoculated plants also showed yellowing, wilting and internal necrosis. *Neoscytalidium novaehollandiae* was re-isolated (100%) from the inoculated seedlings, thus fulfilling Koch's postulates. No symptoms were observed on the mock-inoculated controls.

Neoscytalidium novaehollandiae was described from Australia as a pathogen of *Mangifera indica* (Pavlic *et al*., 2008; Ray *et al*., 2010). This fungus was also associated with dying branches of *Adansonia gibbosa*, *Crotalaria medicaginea, Acacia synchronica*, and *Grevillea agrifolia* in Western Australia (Pavlic *et al*., 2008). Results from the present study showed that *N. novaehollandiae* isolates produce cankers on *Q. brantii* seedlings. This report is the first describing occurrence, molecular characterization and pathogenicity confir-

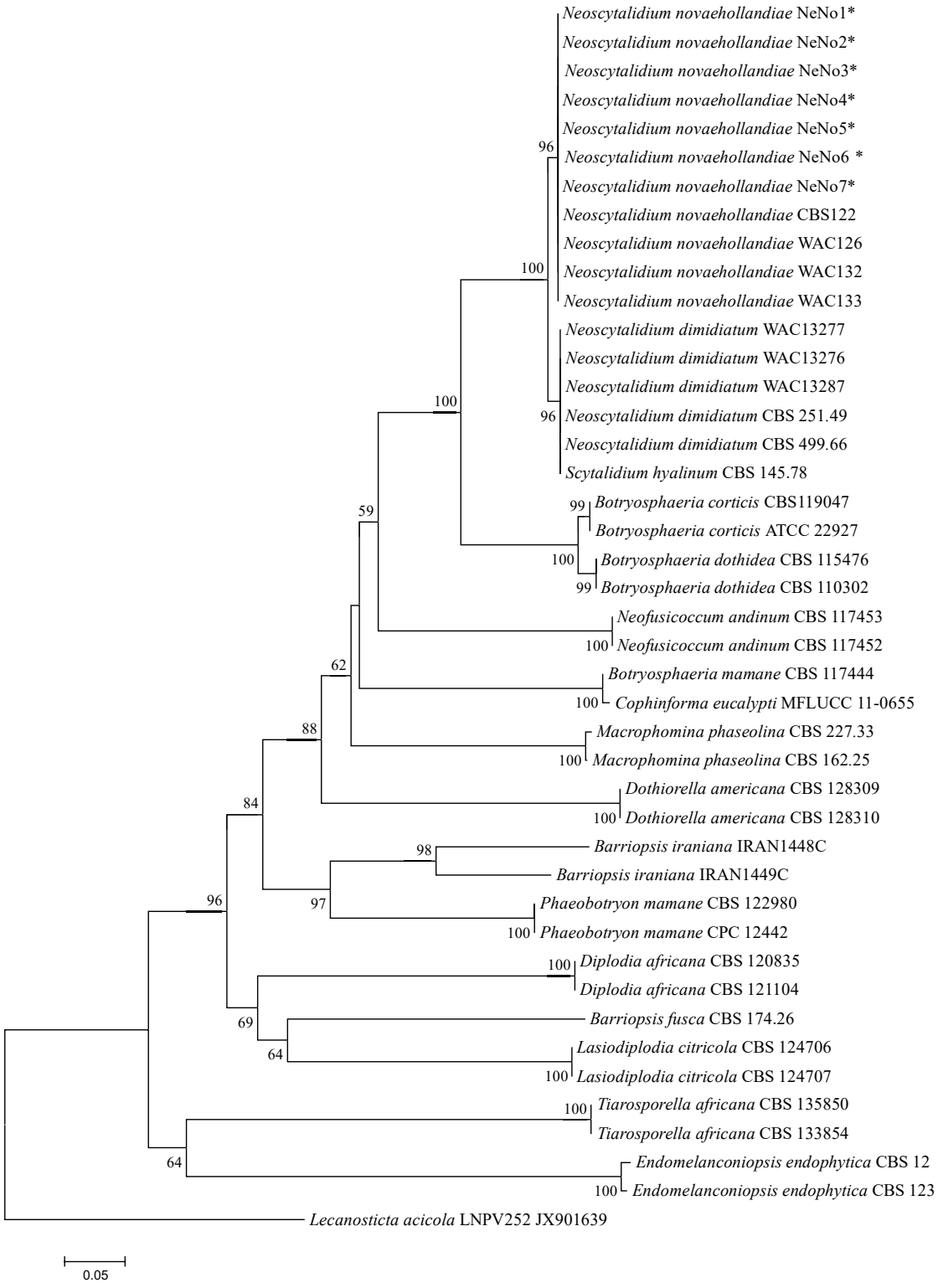

Figure 3. Maximum likelihood phylogram generated in Mega 5 from the alignment of 42 combined internal transcribed spacer (ITS) and translation elongation factor 1-alpha (EF1) gene portion datasets, using the Tamura 3-parameter model with complete deletion gap handling and 1,000-replication bootstrapping. The values associated with each horizontal the line denote bootstrap support for the node. Isolates of *Neoscytalidium novaehollandiae* cluster to a highly supported terminal clade separate from isolates of *N. dimidiatum*. The isolates from Iran are each marked with a star.

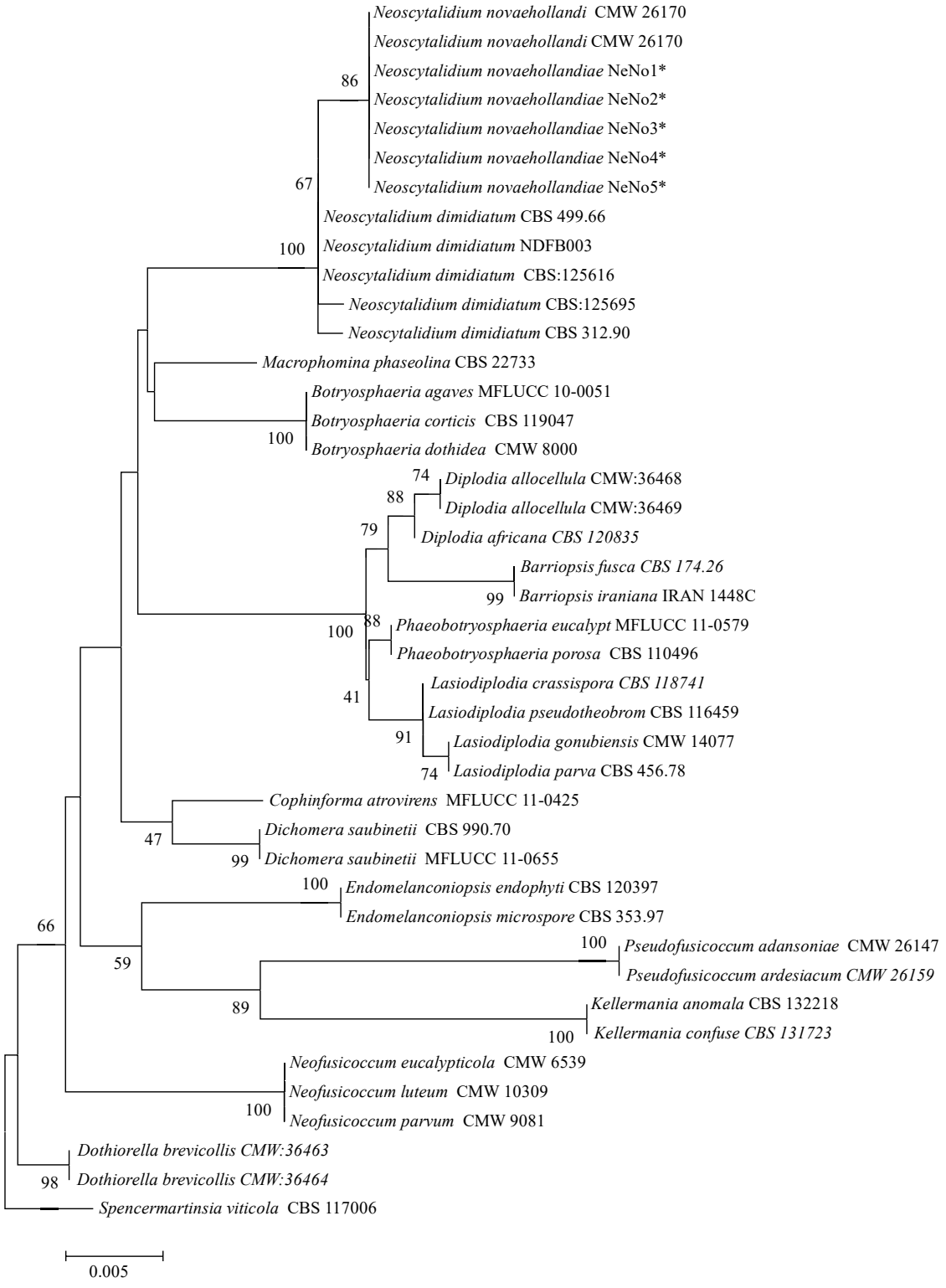

Figure 4. Maximum likelihood phylogram generated in Mega 5 from the alignment of 42 combined internal transcribed spacer (ITS), translation elongation factor 1-alpha (EF1) and nuclear large subunit (LSU) gene portion dataset using Tamura 3-parameter model with complete deletion gap handling and 1000-replication bootstrapping. The values above the line denote bootstrap support for the node. Isolates of *Neoscytalidium novaehollandiae* reside in a highly supported terminal clade separate from isolates of *N. dimidiatum*. The isolates from Iran are marked with an asterisk.

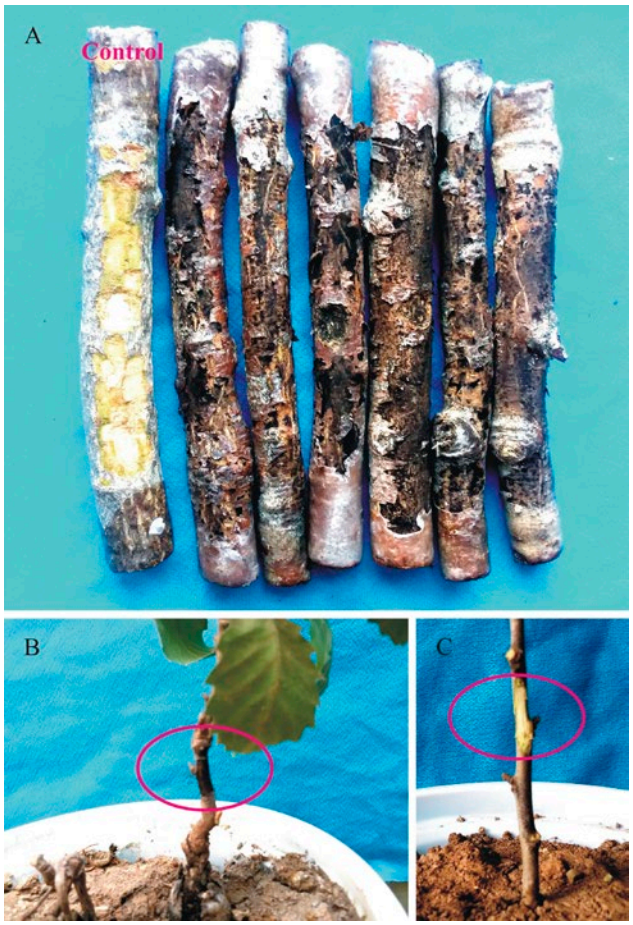

Fig.5. Results of pathogenecity tests performed by inoculating *Neoscytalidium novaehollandiae* isolates from declining Persian oak trees in Persian oak seedlings under laboratory and greenhouse conditions: A. Canker on detached stems, B. Vascular extent of necrotic tissue in an oak seedling 45 days post inoculation with *Neoscytalidium novaehollandiae*, C. Control.

mation for *N. novaehollandiae* causing Persian oak dieback in Iran.

The host range study showed that *N. novaehollandiae* isolates from Iran produced cankers on branches of all other inoculated tree species except for *Pinus*. This information may be useful in the management of this pathogen especially in urban areas.

The minimum, optimum and maximum temperatures for growth of the *N. novaehollandiae* isolates were, respectively, 10, 35 and 40°C, and no growth occurred at 45°C. Similarly, Jamali and Banihashemi (2010) reported that maximum temperature for growth of *N. dimidiatum* was 37°C. *Neoscytalidium* has been reported to be aggressive on drought stressed hosts (Calavan and Wallace, 1954). In spite of the tolerance of Persian oak species to non-optimum temperatures, the emergence of *N. novahollandiae* in *Q. brantii* forests may be related to climate change. Recent temperature increases in the region may have prolonged the host growing seasons, while shortage of rainfall has probably increased the intensity of dehydration in this forest. Desiccation of woody tissues due to drought stress leads to reduced mechanical strength of the bark-wood bonds, and may result in bark cracks that can be invaded by opportunistic fungal pathogens (Bettucci *et al*., 1999).

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