Characterization, Phytopathogenicity and Host Range Studies of Neoscytalidium Dimidiatuml. (Botryosphaeraceae) Associated With Dieback of Lebbeck Trees (Albizia Lebbeck L. Benth) in Saudi Arabia

Khalid E. Hamed (kh.mohammed@qu.edu.sa)
Qassim University https://orcid.org/0000-0002-5278-0104
Abdullah Alsoim
Qassim University College of Agriculture and Veterinary Medicine
Ahmed Rogaibah
Qassim University College of Agriculture and Veterinary Medicine

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Abstract

A survey in 2016 showed that more than 80% lebbeck trees inside the main campus of Qassim University were wilted and dead. Symptoms of dieback, root rot, stem cankers and decline were observed in the trees. The trunks exhibited black masses of spores which soon spread to other, healthy trees. A fungus, having arthroconidial and asexual synanamorph characteristics, and was identified as *Neoscytalidiumdimidiatum* KSA of the class Coelomycetes within the family Botryosphaeraceae and was subsequently isolated from the infected lebbeck trees. Six-year-old lebbeck seedlings were inoculated with the N. dimidiatum KSA isolate. Symptoms of chloroses followed by dropping leaves appeared four weeks after inoculation. The fungus re-isolated from the infected seedlings expressed the same morphological characteristics on the culture media as the N. dimidiatum KSA isolate. A host range study involving six different tree species were inoculated under growth chamber conditions using the identified isolate of the N. dimidiatum KSA fungus. Four weeks after the inoculation, three of these species exhibited wilting and died. To the best of our knowledge, this study is the first to report on N. dimidiatum in Saudi Arabia.

Introduction

The genus *Albizia*includes about one hundred and fifty species, mostly trees and shrubs native tothe tropical and subtropical regionsof Asia and Africa{23}.*Albizia lebbeck* is partiallyidentifiedwith deciduous and semi-deciduous forestswithin Asia from eastern Pakistan through India to Sri Lanka and Myanmar. The tree has been introduced as an ornamental and plantation tree throughout tropicaland northern subtropical regions which includeCentral America, Colombia, Venezuela and Brazil. {13}.

*Albizia lebbeck*is a fast-growing, medium-sized deciduous tree with nitrogen fixation and assimilation capabilities. The treehasan umbrella-shaped crown of thin foliage and a smooth, finely fissured trunk withgreyish-brown bark. Depending on site conditions, annual crown cover increases can spread from 0.5 to 2.0 m.Individual trees attain an average maximum height of18 – 25 m and 50 – 80cm d.b.h.The species grows well at altitudes up to 1500 m a.s.l. in sites receiving 500 – 2500 mm annual rainfall and it can tolerate both light forest and drought conditions. Although it grows poorly in heavy clay soils, it tolerates saline, sodic or lateritic soils. The tree developswell in moist,well-drained soil. Its leaves, seeds, bark and roots are all used in traditional Indian medicine {23}for treating various kinds of ailments. The bark is used for toothache, piles, diarrhoea and diseases of the gum. Decoctions of the leaves and bark are used to treat bronchial asthma and other allergic disorders {2}. In their folk medicine, the Segen people of Ethiopia use the bark of the tree in the management of salmonellosis. Various studies have indicated thatdifferent parts of the barkcovering different regions of the stem exhibitdifferent activities{25}.

Many years ago, *A. lebbeck* was introduced to central Saudi Arabiafrom India and it has adapted well to the severe environmental conditions {14}.The tree is characterized by its resistance to heat and drought andtolerance of harsh environments.

The *Botryosphaeriaceae*include various morphologically diverse fungi that act as pathogens, endophytes or saprobes, mainly on woody hosts. They are found in all geographical and climatic areas of the world, with the exception of the polar regions. Their persistent association with plant diseases has stimulated substantial interest in these fungi, much of which has been focused on the systematics of species and genera{27}.

Neoscytalidium hyalinum is a plant fungus belonging to the family *Botryosphaeriaceae*. Nattrass{24} first specifiedit by the name *Hendersonula toruloidea*.In 1970, Gentles and Evans {16} reported dermatomycosis in patients from tropical regions, followed byCampbell and Mulder {3}, who presented a new species,*Scytalidium hyalinum*,which was similartothat causing theclinical lesions.Intense discussions on nomenclature have been conducted starting with theseearly descriptions of arthroconidia and pycnidial synanamorphs. Sutton and Dyco{38} changed*H. toruloidea* to *Nattrassiamangiferae* for those mycelial synanamorphs named *Scytalidiumdimidiatum.* Farr et al. {15},using phylogenetic analysis,concurred on *Nattrassia mangiferae.* Furthermore,they presented the name. *Fusicoccumdimidiatum* to replace *S. dimidiatum*. In a revision of the taxonomy of *Botryeospheraceae*, Crous et al.{5} proposed the genus *Neoscytalidium*to replace*S. dimidiatum*with*N. dimidiatum*.

A genus wasproposed by Crous et al. {5} for a fungusrecently reported in Brazil that forms *Scytalidium*-like synanamorphs in the aerial mycelia and *Fusicoccum*-like conidia in the pycnidia{20}.A short time ago, the species*N. hyalinum*was reassigned as*N. dimidiatum* because *S. hyalinum* was phylogenetically indistinguishable from *N. dimidiatum*. Therefore, the older designationwas considered {27}.*Hyalinum* might be conspecific and a new name (*N. dimidiatum*var. *hyalinum)* has been suggested {21}.

Diseases associated with this fungus are reported to be more common in tropical countries. It has been associated with freeze-damaged branches of citrus spp. in California, and appears to be a wound pathogen of this host. In Italy,it causes shoot blight, canker and gummosis disease of citrus {28}{29}.

*Neoscytalidium hyalinum*affects abroad range of plant species, causing branch wilting, cancer, dieback and death of treesPunithalingam and Waterson {31}; Reckhaus {33}; Elshafie and Ba-Omar {12}.Initially, the fungus was reported under the name*Nattrassia mangifera* on mango trees in India, then in citrus trees in California{38} and later, the fungus was found to cause cancer on Pacific madrone (*Arbutus menziesii*)in the USA {11}. Dieback symptoms on mango trees (*Mangifera indica*) and *Ficus carica* in Australia were attributed to *N.dimidiatum*{32}.

The same pathogen was recently reported on lebbeck trees(*A. lebbeck)*,*Delonix regia*, *Ficus carica*, Ficus spp., *Peltophorum petrocarpum* and *Thespesia populena* in the Sultanate of Oman {12}. The same symptoms were observed onlebbeck trees inside the main campus of Qassim University in Buraydah, Central Saudi Arabia.

The aim of this study was to identify and characterize the *Botryosphaeriaceae* occurring on *A.lebbeck* inSaudi Arabia using themorphology of the anamorph stages,PCR-RFLP analysis, DNA sequence comparisons, host range studies and the pathogenicity of these fungi.

Materialand Methods

Symptomatology, sample collection and isolation

The branches and main stems of *A. lebbeck*exhibitedextensive dieback, decline and cracking as the main symptoms of the samplescollected throughout the surveyconducted on the main campus of Qassim University in Buraydah in central Saudi Arabia. The dieback symptomson the lebbeck trees were predominant over 80% of thesurveyed area. Small specimens (4 – 5 mm) from the bark of the main stems and live tissue from the brancheswere collectedin polythene bags and transported to the phytopathology laboratory of theQassim University College of Agriculture and VeterinaryMedicine for initial fungal isolation and further analysis. In addition, pure cultures of the fungus were sent to the Leibniz Institute DSMZ – German CollectionofMicroorganisms and Cell Cultures, Braunschweig, Germany, for molecular analysis and deposition. Thesurfacesof thespecimenswere disinfected for 1 min in 70% ethanol followed by3 min in 1% sodium hypochloride andthereafter washed thoroughly two times withrenewedsterile distilled water and dried on sterile tissues.Using sterile forceps, the pieces were placed onto sterilized disposable petri dishescontainingautoclaved potato dextrose agar (PDA, Oxoid, UK) to which was added 0.5 g/L streptomycin sulphideand thenincubatedat ± 25°Cfor 72 h.

Morphological analysis

The isolate was then placed in petri dishes containing autoclaved 2% water agar (WA Agar, Oxoid, England)using a sterile casuarina needle and incubated for three weeks at ± 25°Cto encourage sporulation of the culturesfor conidial characteristics. In order to identify the species, the variance of morphological characteristics were detected using a light microscope (OLYMPUS CX31)equipped witha digital camera (OLYMPUS EVOLT330) and images were taken. The isolate was deposited in the Leibniz Institute DSMZ – German CollectionofMicroorganisms and Cell Cultures, Braunschweig, Germany, under the accession number DSM 104095.

Pathogenicity tests

An identified isolate of *N.dimidiatum*KSA based on PCR, anamorph morphology and DNA sequence comparisons was used for thepathogenicity trial to fulfil Koch's postulates.The 7-day-old incubated isolate grown on aPDA medium at 25°C was used for inoculations.Six-month-old lebbeck tree seedlings were chosen for pathogenicity tests under growth chamber conditionsof 28°C and 15 h daylight. The height of the trees was approximately 90 cm and the diameters of the main stems approximately 10 mm. The seedling trees were allowed toacclimatizeunder thegrowth chamber conditionsfor one month before the inoculations were performed.A sterile cork borer was used to pick up6-mm-diameterdiscs of the PDA medium with the fungal growth of*N.dimidiatum*KSA isolates including mycelia. The inoculation was performed on thestems of 10 trees by removing the bark 5 cm above soil levelto disclose the cambium.Five trees were also inoculated with a sterile PDA medium and kept as controls. The areas of inoculation were wrapped tightly with plastic parafilm(Pechiney Plastic Packaging) to prevent drying and contamination. Six weeks post inoculation, the bark of the inoculated trees was removed from 5 cm

above and below the wounded area and small samples from all inoculated and non-inoculated trees wereplated on a PDA medium for fungal isolation.

Host range studies

The tree species used for the host range studies were selectedbased on ecological importance to the environment of Saudi Arabia andtheir potential susceptibility to the pathogen. Ten healthy seedling treesof *Ficus infectoria, Moringa oleifera, Casuarina cunninghan, Enterlobium cyclocorpum, Eucalyptus tereticornis* and*Ficus nitida* agedabout six monthswere collected from the forests nurseryofthe College of Agriculture and Veterinary Medicine farm. The seedling trees were grown in plastic potsfilled with a 2:1 mixture of sand and clay soil. The seedling trees were maintainedunder growth chamber conditions at 26^{o}C and 30–60% relative humidity and irrigated once a week. Eight out of the ten trees of each species were inoculated using the identified isolate of *N.dimidiatum*KSA as described previously and the remaining two seedlings of each species were left uninoculated for comparison. Monitoring for the development of symptoms was done weekly. Fungal re-isolation was carried out to verify Koch's postulates.

DNA extraction and PCR sequencing for identification of isolates

Pure cultures were maintained on 2% malt extract agar and harvested directly from the plates. Genomic DNA was extracted using the MasterPure™ Yeast (Epicentre, for Fungi and Yeasts) DNA Isolation Kit. The strain was PCR amplified and sequenced, targeting the partial gene of the translation elongation factor alpha 1 using primers EF1-728Fand EF1-986R {4}.

The PCR mixtures contained Taq and PCR buffer (Takara Bio Inc., Kusatsu, Japan), 5pM of each primer (Eurofins),200 µM dNTPs (Roche), 40–200 ng of genomic DNA and nuclease-free water. The PCR was performed with an initial denaturation step for 2 min at 94°C, followed by 25–35 cycles of denaturation for 1 min at 94°C, 45-s primer annealing, and elongation for 1 min at 72°C. The quality of the PCR amplicons was checked in 1.2% agarose gel stained with GelStar (Lonza, Switzerland) under UV light using a 1 kb ladder (Gene Ruler 1, Thermo Scientific, Germany). The amplicons were purified using the QuiaQuick purification kit (Quiagen, Germany). Sequences were generated with an AB 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and the AmpliTaq FS Big Dye terminator cycle sequencing kit. To type strain references when appropriate,all sequences were used as queries in the GenBank and MycoBank sequence similarity search tool BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi] with default stringency and restrictions.

Phylogenetic analysis

Phylogenetic relationships were assessed using the ARB software package (Ludwig et al. 2004). All sequences were aligned using Fast Aligner/ClustalW and Muscle (Edgar 2004) implemented in ARB V1.06. All alignments were thoroughly examined and manually optimized according to primary and

secondary structure information calculated by ARB. Ambiguously aligned nucleotide characters were excluded manually prior to phylogenetic analyses. The jModeltest 2.1.1 (Darriba et al. 2012) was used for the selection of the model of nucleotide substitution that best fit the sequence data, employing the Akaike Information Criterion (Posada and Buckley 2004). Maximum Likelihood analyses were performed with ARB using RAxML(Randomized Accelerated Maximum Likelihood) 7.0.3 (Stamatakis 2006) applying the GTRGAMMAmodel of sequence evolution for the combined data set. Searches were performed with random sequence additions and 100 replicates. Branch support was tested with 1000 replications on bootstrapped data sets.

Results

Symptomatology and fungal isolation

A survey of the lebbeck trees planted as avenue shade on the main campus of Qassim University in Buraydah in central Saudi Arabia revealed that more than 80% of the trees were wilted and dead. Symptoms of dieback, root rot, stem canker and decline were apparent (Fig. 1a). These symptoms included yellowing branches and browning of leaves and subsequent leaf fall (Fig. 1b). Consequently, rapid death of the branches and cracking of the main trunkswere observed (Fig. 1c).A black mass of fungal spores could be observed on the trunk (Fig. 1f)which soon spread by wind or water to other,healthy trees.The bark of the main roots presentedthe same symptoms.The fungus *N.dimidiatum*was isolated from the cracking stems of the ten representative symptomatic lebbeck trees exhibiting dieback. The isolate and its relation to other species of *Botryosphaeriaceae*were later confirmed by DNA sequencing.

Culture characteristics and morphology

The colonies on the PDA media were initially whitish, butgradually turned blackish afterthree days (Fig. 2a). They were flatand attained radial growth with threadlike forms onthe margin in 3–5 days at 25°C.For inducing the sporulation, an identified single spore culture was placed on the surface of 2% water agar medium using a sterile casuarina needle and incubated at 25°C.A chain of cylindrical or spherical arthroconidia appeared on the branched aerial mycelium.Seen under microscopy, the hyaline conidia freed from pycnidia weretruncate at the base,immersed,dark to darkbrown with smooth thick walls, at first aseptate andlater becoming septatewith central dark brown septa(Fig. 2b). Characteristic of the synasexual morph of coelomycetes, the stromatic conidiomata seen were immersed,lately decrepitate, dark brown or black, spherical, 2–3 mm in diameter with thick, black cell layers and irregular outer walls, while the inner walls were thin and hyaline(Figs. 2c, d).

Pathogenicity tests

Theinoculated lebbeck tree seedlings were assessed after tendays post inoculation. The *N.dimidiatum*elicited symptoms similar to those shown on the trees naturally infected. Ten days after inoculation, symptoms were starting to appear on four out of eight stems of the inoculated seedlings.Firstly, leaves and branches developed chlorosis, startingwith old leaves, and subsequent leaf

fall, and four weeks later, all eight inoculated lebbeck tree seedlings werewilted and bareof leaves,while the uninoculated control seedlings showed no symptoms under test conditions. Re-isolation of the fungus from the stems of the infected lebbeck seedlingsperformed on PDA media to accomplish Koch's postulates revealed the fungus to be *N.dimidiatum*(Fig. 2).

Host range

The different host plant species examined exhibited symptoms as follows.In *Ficus infectoria*, all seedling treesinoculated with *N. hyalinum* showedchlorosis in the leaves followed by necrosis two weeks after inoculation. Canker of the main stem extending to the crown was observed. Thereafter,all the seedlings were completely wilted. At the inoculation site under the bark,a sooty coat of black spores was found coming off in layers (Fig. 3a). In *Moringa oleifera*, symptoms were seen ten days after inoculation.Chlorosis of the leaves and then leaf fall developed in 50% of the inoculated seedlings. A month later, 80% of the seedlings were completely wilted, while the controls remainedfree of any symptoms (Fig. 3b).In *Enterlobium cyclocorpum*, leaves of the inoculated seedlings initially became pale and developed chlorosis and necrosis followed byleaf dropthree weeks after inoculation.In five to six weeks, 75% of the seedlings inoculated under the bark of the stem were completely wilted, whereas the control seedlings did not show any symptoms (Fig. 3c). The*Casuarina equstifolia, Eucalyptus tereticornis* and *Ficus nitida*seedlings did not produce any symptoms under test conditions and were indistinguishable from the uninoculated seedlings (Fig. 3d).

Re-isolation from the infected seedlings on PDA media confirmed that *N. hyalinum* was the prevalent fungal pathogen isolated; however, the uninoculated seedlings

DNA sequencing and phylogenetic analysis

Sequencing of the elongation factor alpha 1 gene region and phylogenetic analysis confirmed that our isolate was*N. dimidiatum* (Penz.) Crous & Slippers, a member of the family *Botryosphaeriaceae* (Fig. 4).Some of the closest BLAST hits for our isolate were identified as *N. hyalinum*. According to Huang et al. {18}, *N. hyalinum* is synonymous with *N. dimidiatum* due to the con-specificity of the two.

Discussion

When first observed, it was striking that most of the lebbeck trees of different ages planted on the main campus of Qassim University at Al-Mulayda (Buraydah), Qassim, central Saudi Arabia, were wilted or dead. Initially, the trees exhibited symptoms ofyellowing and chlorosis on branches and leaves, which turned brown and subsequently suffered leaf fall followed by dieback, stem canker and decline (Fig. 1a). Consequently, quick death of the branches and cracking of the main trunkswere observed. The trunksexhibited a black mass of fungal spores through the cracking bark (Fig. 1c). The symptoms described here are identical to those reported on lebbeck trees in the Sultanate of Oman by Elshafie and Ba-Omar {12} and likewise byAbbasher et al. {1}in ficus trees, Giha {17}in mango trees *(Mangifera indica*), and El Trefee{10}in the fruit orchards of Sudan.

In the present study, for the first time, a new isolate of *N.dimidiatum*KSA wasisolated from infected trees andreported as causing dieback and mortality of lebbeck trees in Saudi Arabia based on symptomatology, phytopathogenicity, host range studies, DNA sequencing, phylogenetic analysis and morphological characteristics of the coelomycetous asexual morph with stromatic dark brown to black, black and erumpent conidiomata, and ellipsoidal to oval and hyaline conidia. The fungus *Natrrassiamangifra* has a wide geographical range in Africa, Asia, and North and South America {31}. The fungus was first recorded by Nattrass{24}as causing rapid death on deciduous trees in Egypt, by Sutton and Dyko {38}as causing the same on mango trees in India and theUnited States.

Neoscytalidium dimidiatum,belonging to the *Botryosphaeriaceae*family, has been reported to have a wide host range, including apple, banana, citrus, fig, and mango {37}; {29}; {32}; {27}.The isolate *N. hyalinum* reported herewas characterized by 1–2 septa, brown conidia in the coelomycete morph and holothallic fragmentation of undifferentiated hyphae on potato dextrose agar. In this study,*N.dimidiatum*had thedimensions of conidia, arthroconidial sporesand conidiogenous cells typicalof descriptions of this species in previous studies {26}; {27}.

Based on phylogenetic analysis of the elongation factor alpha 1 gene region of the DSM104095 isolatewith 70 othersequencesof the members of *Botryosphaeriaceae* available in the NCBI/GenBank, it wasdemonstrated that*N. dimidiatum*together with the other fiveisolates in the genus, specifically*N. dimidiatum*,constitute a separate clade in the tree reconstruction (Fig. 4). All sequences of isolates within this cluster are more closely related to each other than to other members of *Botryosphaeriaceae*or other species of the genus *Neoscytalidium*. Some of the closest BLAST hits for our isolate were identified as*N. hyalinum*.Regarding*N. hyalinum*, Crous et al.{5}commented that *Scytalidium*had a polyphyletic feature and introduced *Neoscytalidium* to transform*Scytalidium dimidiatum*to *N. dimidiatum*.According to Huang et al. {18},*N. hyalinum* is synonymous with *N. dimidiatum* due to the con-specificity of the two isolates.Madrida et al.{21} suggested that *S. dimidiatum* and *S. hyalinum* could be synonymous,which also agreed with Phillips et al. {27}, who considered them as members of the same species and joined them under the name*N. hyalinum*.

*Neoscytalidium dimidiatum*is considered an important threat to the sustainability of *A. lebbeck*trees and other flora in Saudi Arabia. Slippers and Wingfield {35} and Slippers et al.{34}reported that this funguswas capable of infecting native and introduced or cultivated hosts and seemed to move easily between different regions and provinces. The results of this study can provide a base for further work on managing the diseases caused by the *Botryosphaeriaceae* in Saudi Arabia.

El Gamal et al.{9} reported that thelebbeck trees (*A. lebbeck*) imported from India to Saudi Arabia as an ornamental tree were well acclimatized to the hot environmental conditions of the central region of Saudi Arabia. It was clear that water stress, high temperatures and wind had influenced the incidence and severity of the disease {22}. The extreme climate of the central region of Saudi Arabia in addition to stress factors may have contributed to the susceptibility of the lebbeck trees to the dieback disease associated

with *N. dimidiatum*. This is the first report of *N. dimidiatum*associated with lebbeck dieback in Saudi Arabia.

Over the next 50–100 years, climatic change is expected to exert a passive influenceon the extent of forest land in Saudi Arabia. An increase in the frequency of natural phenomena such as drought, sand storms, fire and flood will lead to increased dieback in forests and woodlands, spread of diseases, changesin type and number of species, a drop in productivity and a reduction in biodiversity {6}.

Declarations

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Author contributions

KEH, ASA and AAR Conceptualiza-tion; KEH conceived the idea. KEH, ASA and AAR collected all the materials. KEH, ASA and AAR wrote the paper, and KEH, ASA and AAR revised the manuscript.

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Conflict of interest

All authors declare that they have no conflict of interest.

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Figures

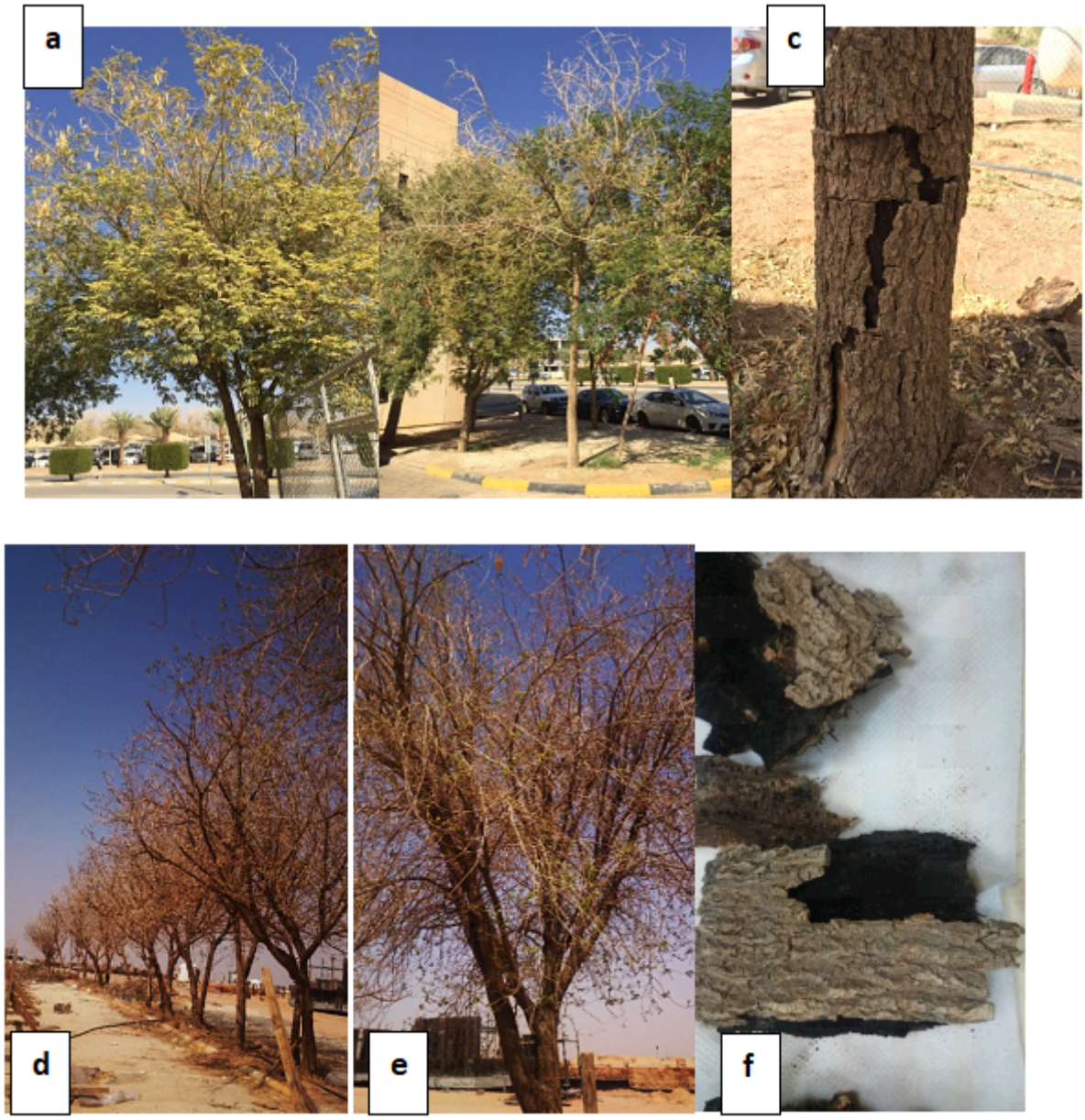

Figure 1

aBranches of A. lebbeckinfectedwith N.dimidiatumKSA.bMain stem infectionof A. lebbeck by N.dimidiatumKSA.cCracking of the trunk;d – e .f Black fungalstructures with a black mass ofsporeserupting from the bark of the tree.

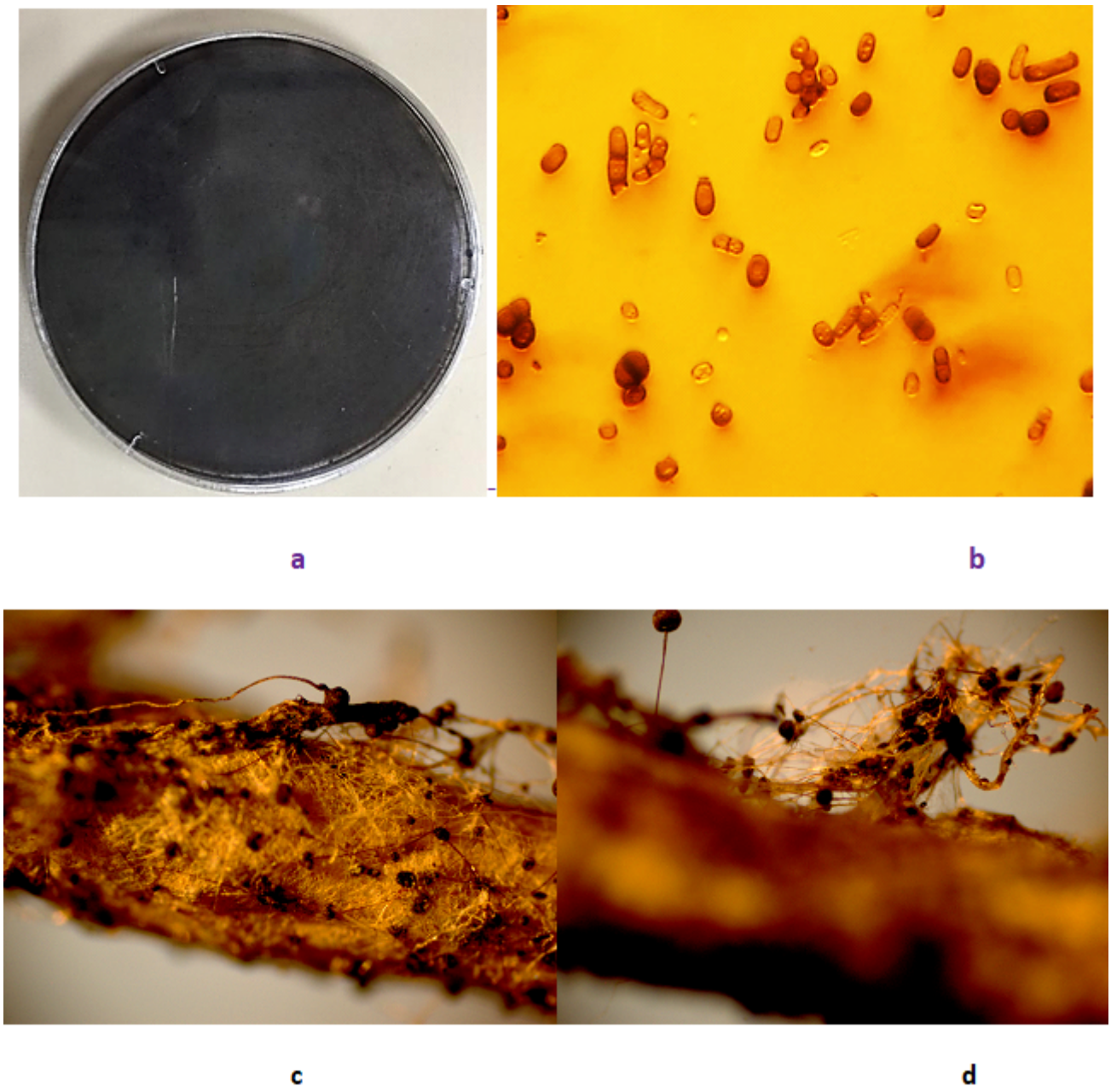

Figure 2

NeoscytalidiumdimidiatumKSA(a)Culture of N. hyalinumon PDA after three daysincubation at 25°C. (b)Immature hyaline pycnidial conidia, fragmented and with central band.(c)Conidiomata formed on casuarina needle in culture.(d)Arthricchains of coelomycetousconidia. remained free of any symptoms.

Figure 3

aHost rangesymptoms of wilting in Ficus infectoria.bDiseased plants in the field; c – d Plants wilting.e – fDead plants. g –kNecrotic symptoms on the vascular system.

Figure 4

Maximum Likelihood phylogenetic tree calculated from the analysis of the combined elongation factor alpha 1 gene region sequence data for 71species and isolates of Botryosphaeriaceae. Bootstrap values for maximum likelihoodgreater than 80% areindicated above the nodes. IsolateDSM104095from this studyis indicated in bold. Isolates of the fungus within one clade are indicated in the box.