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

Research Article

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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 *Neoscytalidium dimidiatum* 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 to the tropical and subtropical regions of Asia and Africa. *Albizia lebbeck* is partially identified with deciduous and semi-deciduous forests within Asia from eastern Pakistan through India to Sri Lanka and Myanmar. The tree has been introduced as an ornamental and plantation tree throughout tropical and northern subtropical regions which include Central America, Colombia, Venezuela and Brazil.

*Albizia lebbeck* is a fast-growing, medium-sized deciduous tree with nitrogen fixation and assimilation capabilities. The tree has an umbrella-shaped crown of thin foliage and a smooth, finely fissured trunk with greyish-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 of 18 – 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 develops well in moist, well-drained soil. Its leaves, seeds, bark and roots are all used in traditional Indian medicine 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. In their folk medicine, the Segen people of Ethiopia use the bark of the tree in the management of salmonellosis. Various studies have indicated that different parts of the bark covering different regions of the stem exhibit different activities.

Many years ago, *A. lebbeck* was introduced to central Saudi Arabia from India and it has adapted well to the severe environmental conditions. The tree is characterized by its resistance to heat and drought and tolerance 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 specified it by the name *Hendersonula toruloidea*. In 1970, Gentles and Evans \(^{16}\) reported dermatomycosis in patients from tropical regions, followed by Campbell and Mulder \(^{3}\), who presented a new species, *Scytalidium hyalinum*, which was similar to that causing the clinical lesions. Intense discussions on nomenclature have been conducted starting with these early descriptions of arthroconidia and pycnidial synanamorphs. Sutton and Dyco\(^{38}\) changed *H. toruloidea* to *Nattrassia mangiferae* for those mycelial synanamorphs named *Scytalidium dimidiatum*. Farr et al. \(^{15}\), using phylogenetic analysis, concurred on *Nattrassia mangiferae*. Furthermore, they presented the name *Fusicoccum dimidiatum* to replace *S. dimidiatum*. In a revision of the taxonomy of *Botryosphaeraceae*, Crous et al. \(^{5}\) proposed the genus *Neoscytalidium* to replace *S. dimidiatum* with *N. dimidiatum*.

A genus was proposed by Crous et al. \(^{5}\) for a fungus recently 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 designation was 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 a broad range of plant species, causing branch wilting, cancer, dieback and death of trees Punithalingam 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 on lebbeck 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* in Saudi Arabia using the morphology of the anamorph stages, PCR-RFLP analysis, DNA sequence comparisons, host range studies and the pathogenicity of these fungi.
Material and Methods

Symptomatology, sample collection and isolation

The branches and main stems of *A. lebbeck* exhibited extensive dieback, decline and cracking as the main symptoms of the samples collected throughout the survey conducted on the main campus of Qassim University in Buraydah in central Saudi Arabia. The dieback symptoms on the lebbeck trees were predominant over 80% of the surveyed area. Small specimens (4 – 5 mm) from the bark of the main stems and live tissue from the branches were collected in polythene bags and transported to the phytopathology laboratory of the Qassim University College of Agriculture and Veterinary Medicine for initial fungal isolation and further analysis. In addition, pure cultures of the fungus were sent to the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, for molecular analysis and deposition. The surfaces of the specimens were disinfected for 1 min in 70% ethanol followed by 3 min in 1% sodium hypochloride and thereafter washed thoroughly two times with renewed sterile distilled water and dried on sterile tissues. Using sterile forceps, the pieces were placed onto sterilized disposable petri dishes containing autoclaved potato dextrose agar (PDA, Oxoid, UK) to which was added 0.5 g/L streptomycin sulphide and then incubated at ± 25°C for 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°C to encourage sporulation of the cultures for conidial characteristics. In order to identify the species, the variance of morphological characteristics were detected using a light microscope (OLYMPUS CX31) equipped with a digital camera (OLYMPUS EVOLT330) and images were taken. The isolate was deposited in the Leibniz Institute DSMZ – German Collection of Microorganisms 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 the pathogenicity 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 conditions of 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 to acclimatize under the growth chamber conditions for one month before the inoculations were performed. A sterile cork borer was used to pick up 6-mm-diameter discs of the PDA medium with the fungal growth of *N. dimidiatum* KSA isolates including mycelia. The inoculation was performed on the stems of 10 trees by removing the bark 5 cm above soil level to 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 were plated on a PDA medium for fungal isolation.

**Host range studies**

The tree species used for the host range studies were selected based on ecological importance to the environment of Saudi Arabia and their potential susceptibility to the pathogen. Ten healthy seedling trees of *Ficus infectoria, Moringa oleifera, Casuarina cunningham, Enterlobium cyclocorpum, Eucalyptus tereticornis* and *Ficus nitida* aged about six months were collected from the forests nursery of the College of Agriculture and Veterinary Medicine farm. The seedling trees were grown in plastic pots filled with a 2:1 mixture of sand and clay soil. The seedling trees were maintained under growth chamber conditions at 26°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 EF1-728F and 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 GTRGAMMA model 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 trunk were 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 presented the 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, but gradually turned blackish after three days (Fig. 2a). They were flattand attained radial growth with threadlike forms on the 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 were truncate at the base, immersed, dark to dark brown with smooth thick walls, at first aseptate and later becoming septate with 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

The inoculated lebbeck tree seedlings were assessed after ten days 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, starting with 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 seedlings performed 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
trees inoculated with *N. hyalinum* showed chlorosis 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 remained free of any
symptoms (Fig. 3b). In *Enterolobium cyclocarpum*, leaves of the inoculated seedlings initially became pale
and developed chlorosis and necrosis followed by leaf drop three 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 nitidaseedlings 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 of yellowing 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 trunk were observed. The
trunk exhibited 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 by Abbasher 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 was isolated from infected trees and reported 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 _Nattrassiamangifra_ 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 the United 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 here was 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 the dimensions of conidia, arthroconidial spores and conidiogenous cells typical of descriptions of this species in previous studies (26); (27).

Based on phylogenetic analysis of the elongation factor alpha 1 gene region of the DSM104095 isolate with 70 other sequences of the members of _Botryosphaeriaceae_ available in the NCBI/GenBank, it was demonstrated that _N. dimidiatum_ together with the other five isolates 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 fungus was 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 the lebbeck 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 \textit{N. dimidiatum}. This is the first report of \textit{N. dimidiatum} associated with lebbeck dieback in Saudi Arabia.

Over the next 50–100 years, climatic change is expected to exert a passive influence on 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, changes in type and number of species, a drop in productivity and a reduction in biodiversity \cite{6}.

\section*{Declarations}

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\subsection*{Author contributions}

KEH, ASA and AAR Conceptualization; 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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\subsection*{Compliance with ethical standards}

\subsection*{Conflict of interest}

All authors declare that they have no conflict of interest.

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Figures
Figure 1

a Branches of *A. lebbeck* infected with *N. dimidiatum*.
b Main stem infection of *A. lebbeck* by *N. dimidiatum*.
c Cracking of the trunk.
d – e .f Black fungal structures with a black mass of spores erupting from the bark of the tree.
Figure 2

Neoscytalidium dimidiatum KSA

(a) Culture of N. hyalinum on PDA after three days incubation at 25°C.
(b) Immature hyaline pycnidial conidia, fragmented and with central band.
(c) Conidiomata formed on casuarina needle in culture.
(d) Arthric chains of coelomycetous conidia. remained free of any symptoms.
Figure 3

a Host range symptoms of wilting in Ficus infectoria. b Diseased plants in the field; c – d Plants wilting. e – f Dead plants. g – k Necrotic 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 71 species and isolates of Botryosphaeriaceae. Bootstrap values for maximum likelihood greater than 80% are indicated above the nodes. Isolate DSM104095 from this study is indicated in bold. Isolates of the fungus within one clade are indicated in the box.