https://doi.org/10.17221/82/2022-PPS

Morphological and molecular characterization of *Neoscytalidium* isolates that cause canker and dieback in eucalyptus and chinaberry trees in Iraq

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Citation: Abdulrahman D.N., Haleem R.A. (2023): Morphological and molecular characterization of *Neoscytalidium* isolates that cause canker and dieback in eucalyptus and chinaberry trees in Iraq. Plant Protect. Sci., 59: 92–105.

Abstract: *Neoscytalidium dimidiatum* isolates are the most pathogens associated with sooty canker and dieback in the stem and twigs of eucalyptus and chinaberry trees in the Kurdistan region of Iraq. Young trees showed branch dieback and yellowing leaves. The symptoms were further developed to sooty canker and dieback appeared on the main branch and trunk. *N. dimidiatum* colonies on potato dextrose agar were dense white at first and became dark gray to black within seven to 10 days. Arthric dark brown conidia (6.6 × 4.3 µm) were observed in the chain of mycelium. The color of the colonies was white at the beginning, then eventually turned greenish in seven days, and finally became black. The fungus produced white to olivaceous aerial mycelium with chains of chlamydospores and arthroconidia. Conidia were initially hyaline, ellipsoidal to globose, 4.1–9.8 × 2.8–3.5 m, with muriform septa. The inoculated eucalyptus and chinaberry seedlings displayed necrosis streaks along with the barks and xylem of the inoculation points. Combined dataset of internal transcribed spacer, nuclear ribosomal large subunite, and beta tubulin 2a using maximum likelihood and maximum parsimony analysis support the monophyletic on *N. dimidiatum* isolates from Iraq [*N. hyalinum* (No. B21), and *N. novaehollandiae* (No. B22)]. This is the first time to report *N. dimidiatum* on chinaberry in Iraq.

Keywords: sooty canker; *Neoscytalidium* isolate; *N. dimidiatum*; phylogenetic lineages

Eucalyptus belongs to the Myrtaceae family and has become the main source of different industrial applications such as the production of paper, furniture, energy, charcoal, and housing in the last century. The *Eucalyptus* genus includes about 700 species (Brooker 2000). Several *Eucalyptus* species are planted in Iraq; however, the most common species are *E. microtheca* Muell and *E. calmaldulensis* Dehn (Al-Iryani 1998). Botryosphaeriaceae, a family of sac fungi (Ascomycetes), is the typical representative of Botryosphaeriales order. The fungi of this family are usually connected with cankers and dieback in shrubs and woody plants (Bush 2018). According to Crous et al. (2006), the genus *Neoscytalidium* is a genus of plant pathogenic fungus causing cankers and dieback on woody plants all over the world. In Kurdistan region, several symptoms have been observed in the eucalyptus

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trees including several leaves discoloration, premature defoliation and die back. *Neoscytalidium dimidiatum*, have been previously recorded on different hosts such as *Adansonia gibbose, Vitis vinifera, Mangifera indica*, *Crotalaria medicaginea*, *Grevillia agrifolia* and *Acacia synchronica* (Pavlic et al. 2008; Ray et al. 2010a)

Although chinaberry (*Melia azedarach L.*) is an ornamental plant with high adaptive/resistance ability to different pests, it can be vulnerable to other pathogenic fungi, such as *Pseudocercospora subsessilis* that causes leaf-spot disease (Seo et al. 2013). In addition, the weak limbs of chinaberry trees vulnerable to severe wind during both summer and winter can be broken. This creates excellent target points for fungal and bacterial infection.

Pathogenicity studies have revealed that *N. dimidiatum* is the most pathogenic Botryosphaeriaceae species (Marques et al. 2013). It can be isolated from the roots of fig shrubs that exhibit dieback signs (Ray et al. 2010b). In the southern California desert, *N. dimidiatum* is connected to a citrus branch canker (Mayorquin et al. 2016). It is essential to know that the name of this species has been changed and corrected recently from *N. hyalinum*, the older classification published by Phillips et al. (2013) to *N. dimidiatum* in 2016 by Huang et al. (2016). According to reports from Turkey and Iran, *N. dimidiatum* has been linked to *Quercus brantii* dieback and grapevine wood canker (Akgül 2019). The *N. dimidiatum* that causes sooty canker on the eucalyptus tree was first isolated and identified by Altememe et al. (2019) in southern Iraq. The isolations of several infected tissues consistently produced a scytalidium-like fungus. However, there is a high possibility of finding more isolates that can infect eucalyptus trees in the Kurdistan area of Iraq. This is because of the wide spread of this pathogen which infects almost all woody plant hosts examined globally.

It is difficult to identify theses pathogens at the species level (Machouart et al. 2004). Thus, finding efficient, dependable, and reproducible assays to confirm the pathogen species became crucial. Accordingly, PCR-based techniques provide excellent results in a shorter time (less than a week) than the conventional techniques (several weeks or months), allowing for an early preventive reaction to the infections' risks. Unfortunately, the entire *Neoscytalidium* genome data has not been sequenced yet.

For many years, the species identification process in the Botryosphaeriaceae family was poor and inconsistent. However, DNA-based phylogenetics now provides a more accurate substitute technique (Crous et al. 2006; Slippers et al. 2006; Pavlic et al. 2009). Several new species of Botryosphaeriaceae have been discovered, identified, and described in recent years using genetic data in combination with morphological descriptions (Phillips et al. 2005, 2013; Crous et al. 2006; Slippers & Wingfield 2007; Pavlic et al. 2008; Lawrence et al. 2017).

Since the genetic information of this genus is extremely limited, the detection of *Neoscytalidium* species is confined to a few rRNA loci, few dispersed exonic areas, or both (Pavlic et al. 2008; Ray et al. 2010b; Bakhshizadeh et al. 2014). Accordingly, it is quite hard to have a complete idea and complete understanding of any unique sequences of these species recently. Internal transcribed spacers (ITS) have been verified as the best marker genes for all fungus classes, and as the recommended technique for providing phylogenetic information (Nilsson et al. 2009). In Iraq, ITS primer was utilized by Al-Shuhaib et al. (2018) to identify two isolates of *Neosytalidium*, *N. dimidiatum,* and *N. novaehollandiae*, from canker-infected fig trees. In addition, Altememe et al. (2019) have isolated *N. dimidiatum* from eucalyptus trees as the cause of sooty canker in Karbala province – Iraq. They confirmed the identification results based on ITS primer. Furthermore, *N. dimidiatum* was isolated from infected Apple tree in Iran (Nourian et al. 2021) and Olive canker in Turkey (Güney et al. 2022) using morphological characteristics as well as phylogenetic analysis of fungal ITS, the translation elongation factor 1(TEF1) and tubulin (Bt2a) gene. Moreover, Crous et al. (2006) and Slippers and Wingfield (2007) replaced *S. dimidiatum* with *N. dimidiatum* depend on the aerial conidia and confirmed by DNA-based phylogenetic position. Pavlic et al. (2008) used ITS and TEF1 to identify seven *Adansonia gibbose* in Western Australia, including *N. novaehollandiae*. Mayorquin et al. (2016) have also utilized ITS to identify *N. dimidiatum*, *N. novaehollandiae,* and other fungal species that cause cankers on citrus trees in California. However, Zhang et al. (2020) and Crous et al. (2021) designated *N. orchidacearum* and *N. novaehollandiae* as synonyms of *N. dimidiatum.*

The objective of this investigation is to distinguish between a virulent *Neoscytalidium* species, with al-

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most similar morphological characters that affect eucalyptus and chinaberry trees in Iraq, using the reliable PCR primers (ITS, LSU, and Bt2a) and understanding the phylogenetic analysis of these species.

MATERIAL AND METHODS

Sample collection, isolation, and identification of the pathogens. A field survey for sooty canker and dieback diseases on eucalyptus and chinaberry trees was conducted during fall season of 2020 and 2021. The infected trees were selected from all provinces of the Kurdistan region-Iraq (Erbil, Dohuk, Sulaymaniyah, and Hallabjah). The trees were considered as infected or possessing the disease if they showed one or more of the following symptoms: necrosis, chlorosis, dieback on twigs and/or branches, canker, wilting of foliage, and the presence of a black sooty layer of conidia under the bark.

A total of 345 eucalyptus and 375 chinaberry samples were collected and places in a plastic bag and taken to the lab. The samples were cleaned using running tap water for one hour. A small part of tissue located between healthy and infected tissues were selected. Then, it was surface-sterilized by 1% sodium hypochlorite for 1–2 min, washed with sterile distilled water, blotted dry with sterile filter paper, and placed on petri dishes with potato dextrose agar (PDA). Then, the samples were incubated for seven days at 25 °C. After incubation, the fungus was sub cultured and kept as a pure culture on PDA. Conidiomata and conidia formation were stimulated in 2% water agar by autoclaved *Pinus brutia* needles (Smith et al. 1996). According to previous descriptions, fungus species were recognized based on microscopic features (Crouse et al. 2006; Pavlic et al. 2008; Philips et al. 2013).

Pathogenicity tests. The experiment was carried out by inserting 5.0 mm diameter agar plugs with mycelium of a 7-day-old colony of the fungus (20 cm above soil line) into wounds made with a sterile sharp instrument under twig bark to expose the cambium of 2-year-old potted seedlings, with the mycelium facing the cambium. Parafilm was used to seal injection sites to prevent contamination and maintain moisture. The control plants were inoculated with sterile PDA plugs. After six months, the lesions' length that developed beneath the stem bark was measured. Re-isolating the pathogen from all inoculation plants and identifying it by conidial morphology confirmed Koch's hypotheses (Moral et al. 2017).

Extraction of genomic DNA and PCR amplification. DNA of fungus samples was extracted by using Genomic DNA Extraction Mini Kit of Fungi/Yeast (Favorgen Biotech Corp., Taiwan). PCR amplification for ITS, LSU and Bt2a genes was applied in 50 µL of reaction mixture containing; 25 µL of 2x Taq DNA Polymerase Master Mix (Ampliqon, Denmark), 2 µL of forward primer, 2 µL of reverse primer, 17 µL DNase free water and 4 µL DNA template by Gradient PCR thermocycler (PTC-200; Bioresearch, USA).

The DNA was subjected to 35 cycles of denaturation (95 °C for 50 s), annealing (primer annealing at varied temperatures according to primers of genes) for 60 s, and elongation (72 °C for 1 min), with the last elongation step at 72 °C for 10 minutes (Table 1).

The PCR products were separated in 1.5% agarose gel stained with ethidium bromide and visualized under UV light. The expected fragment sizes were determined using a typical 100 bp molecular weight marker.

DNA sequencing and alignment. The amplicons termini were sent for sequencing to Microgen

Table 1. The primers used in the current study for phylogenetic analysis

Oligonucleotide primers		Annealing temperature	Fragment size (bp)	References
ITS	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	55	650	White et al. 1990
LSU	ACCCGCTGAACTTAAGC CGCCAGTTCTGCTTACC	58	1 200	Rehner & Samuels 1995
Bt2a	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	58	495	Glass & Donaldson 1995

Bt2a – beta tubulin 2a; ITS – internal transcribed spacer; LSU – nuclear ribosomal large subunite

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Center laboratories in Korea. The PCR product samples were sequenced using the ABI Prism Terminator Sequencing Kit (ABI Prism 3130XL Genetic Analyzer; Applied Biosystems, USA). FinchTV 14.0 nucleotide gene chromatograms were read and edited. The SeqMan program (DNASTAR Laser Gene, USA) was used to gather sequences, annotate them to resolve ambiguities, and aggregate the consensus sequences of all isolates into a single file (FASTA format). Each isolate of the ITS, LSU, and Bt2a data set was analyzed separately, and the results were compared. The possibility of combining ITS, LSU, and Bt2a data was investigated using a partition homogeneity test with Genius prime software (version 2022.1). Sequences of the accessible allied taxa were obtained from their corresponding GenBank accession number (Table 2). The gene sequences are applied to BLASTV, a web browser that uses the sequence alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which can be found on the NCBI website. This tool compares and aligns laboratory or query sequences with other biological sequences to discover more similarities with other targets. The evolutionary history was inferred by using the Maximum Likelihood and the Maximum Parsimony (MP) approach. The MP tree was built using

Table 2. Strains and NCBI GenBank accession numbers of species used in this study

Species	Isolate ID	GenBank accession number		
		LSU	ITS	Bt2a
Neoscytalidium dimidiatum	Arp2-D	MK813853	MK813852	MK816355
Neoscytalidium hyalinum	B-21	OM278779	MZ363623	OM320802
Neoscytalidium dimidiatum	CBS:125695	KX464051	MH863611	KX465065
Neoscytalidium dimidiatum	DCND14	MT341864	MT323060	MT358407
Neoscytalidium dimidiatum	GCND1	MT341860	MT323056.1	MT358403
Neoscytalidium dimidiatum	GRND4	MT341861	MT323057	MT358404
Neoscytalidium dimidiatum	LYND8	MT341863	MT323059	MT358406
Neoscytalidium dimidiatum	mlty_dg01	MW692367	MW696187	MW847274
Neoscytalidium novaehollandiae	B-22	OM278780	MZ363624	OM320801
Botryosphaeria agaves	MFLUCC	JX646808	JX646790	JX646840
Botryosphaeria dothidea	LPSU	KT189498	KT189495	KT189504
Dichomera versiformis	CBS 118101	DQ377889	KF766154	KF766128
Diplodia africana	CBS 120835	MH874653	MH863094	KF766129
Diplodia bulgarica	CBS 124136	MH874879	MH863355	MT592475
Diplodia alatafructa	CBS 124931	MH874935	MH863427	MT592473
Diplodia intermedia	CBS 124462	MH874896	MH863374	MT592503
Dothiorella santali	MUCC509	EF591941	EF591924	EF591958
Dothiorella americana	CBS 128310	MH876299	MH864852	MT592577
Pseudofusicoccum adansoniae	CBS 122055	MH874715	MH863169	MT592771
Phoma herbarum	CBS 276.37	MH855910.1	GU357792	AY749025
Barriopsis iraniana	IRAN 1448C	KF766318	KF766150	KF766127
Neoscytalidium novaehollandiae	Savur_SL01	MT192449	MT193666	–
Neoscytalidium novaehollandiae	Savur_SL02	MT192450	MT193667	–
Neoscytalidium novaehollandiae	Savur_SL03	MT192451	MT193668	–
Neoscytalidium novaehollandiae	Mlty_01	MT038898	MT041243	–
Neoscytalidium novaehollandiae	Mlty_Ma02	MT195555	MT195553	–
Neoscytalidium novaehollandiae	isolate NeNo1	MH899579	KX370828	–
Neoscytalidium novaehollandiae	NeNo3	MH899581	MH883623	–
Neoscytalidium novaehollandiae	NeNo4	MH899582	MH883624	–

Bt2a – beta tubulin 2a; ITS – internal transcribed spacer; LSU – nuclear ribosomal large subunite

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the Subtree-Pruning-Regrafting (SPR) method with search level 1. The initial trees were made by expanding the search space using random sequences and using the Jukes-Cantor model (Jukes et al. 1969; Nei & Kumar 2000). It is assumed that the evolutionary history of the taxa under study is represented by the bootstrap consensus tree generated from 1 000 replicates. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed (Felsenstein 1985). Initial trees for the heuristic search were automatically generated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances determined using the Jukes-Cantor model and then choosing the topology with the highest log likelihood value. There were 33 nucleotide sequences in this analysis. Positions with less than 50% site coverage were all deleted so that no position could have more than 50% alignment gaps, missing data, or unclear bases (partial deletion option). The final dataset contained 1976 locations altogether. MEGA X was used to perform evolutionary analysis (Kumar et al. 2018).

RESULTS

Collection of fungal isolates. The symptoms of dieback and cankers were noticed on many eucalyptus trees at different locations in Kurdistan region, Iraq. As the disease developed, sooty canker started on the main trunk along with dieback on the main branch (Figure 1).

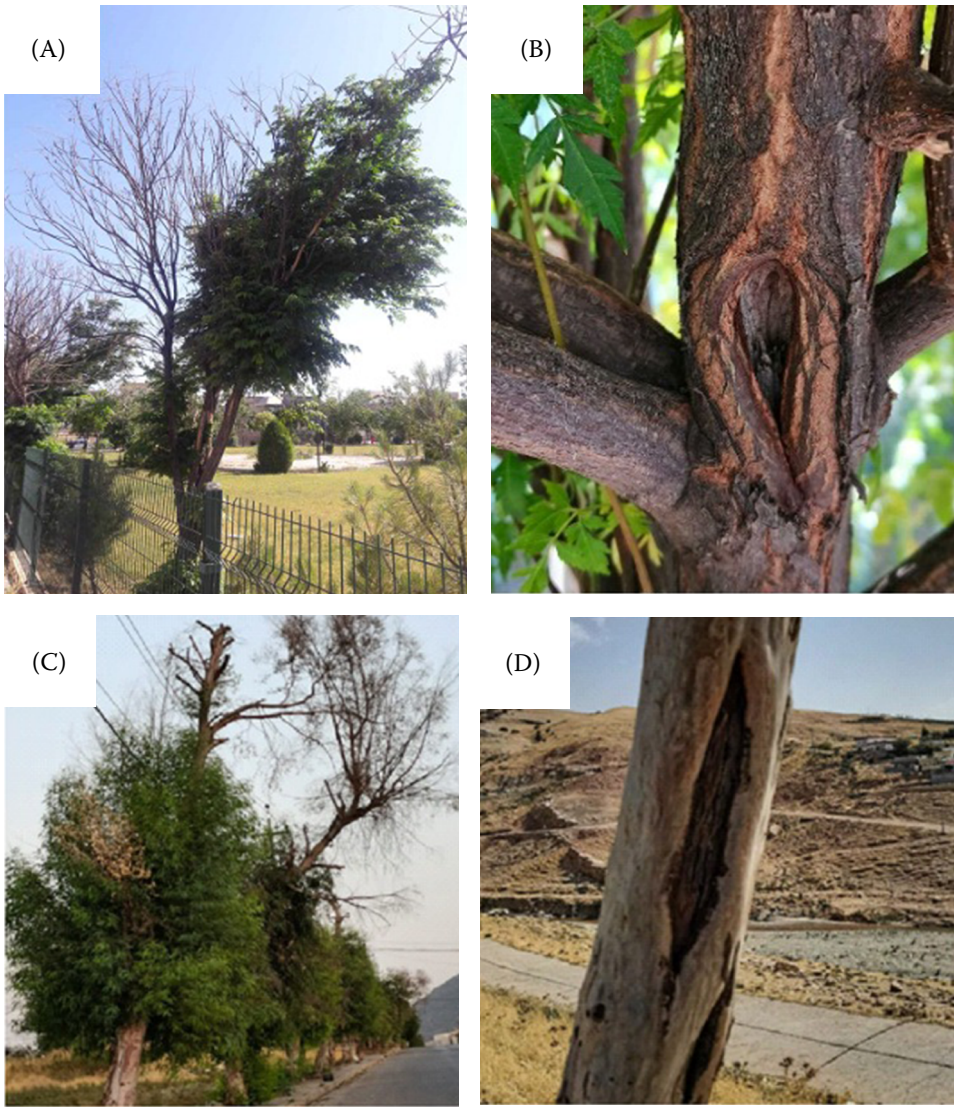

Figure 1. Symptoms of yellowing, dieback and cankers on the tree bark and stem of chinaberry tree (A, B) and eucalyptus tree (C, D)

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In general, the highest disease occurrence was observed in Erbil Province at Koya, Erbil center and Khabat locations (estimated by 98%, 90% and 90% respectively); the lowest infection was observed in Dohuk province at Amide location (37%).

Morphological characterization. After 24–26 h, the affected tissues were developed into fungal mycelia, which were then dispersed to the edge of the Petri plates by day 5 after inoculation. The colonies turned a dark green colour after seven days (Figure 1). A total of 38 isolates were obtained from collected samples. Morphological characterizations of isolated fungi are:

(1) *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers. Isolate No. B-21 [Syn. *N. hyalinum* (C.K. Campb. & J.L. Mulder) A.J.L. Phillips, J.Z. Groenew. & Crous, Studies in Mycology 76: 148 (2013) (MB#805648)].

In the first seven to ten days, colonies were initially powdery white with dense, hairy aerial mycelium, which thereafter became dark gray to black (Figure 2). Conidiophore was absent. Conidia occurred in arthric chain in aerial mycelium, coliform dark brown, thick- walled zero to one septate, 6.6 × 4.3 µm, formed both singly and in arthric chains by hyphal fragmentation. The morphological characteristics were consistent with other descriptions (Phillips et al. 2013; Altememe et al. 2019). This study represents the first record of the identification of *N. dimidiatum* from chinaberry trees in Iraq.

(2) *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers. Isolate No. B-22 [(Syn. *N. novaehollandiae*) *Neoscytalidium novaehollandiae*, Pavlic, T.I. Burgess, M.J. Wingf., Mycologia 100: 862 (2008) (MB#512103) (Pavlic et al. 2008)].

This isolate was identified as *N. dimidiatum* (Syn. *N. novaehollandiae*) according to morphology and phylogenetic analyses. Pavlic et al. (2008) explained that this isolate produces conidia as muriform and *Dichomera* which differentiate it from others. Colonies were initially white and became greenish olivaceous within seven days and black with age. Conidiogenous cell were holoblastic, cylindrical and hyaline. The fungus formed white to olivaceous aerial mycelium containing chains of arthroconidia and chlamydospores). Arthroconidia were aseptate to one septate, thick-walled, hyaline to brown, circular, oval or cylindrical, 7.8–10.5 × 2.3–3.3 µm. Conidia were initially hyaline; ellipsoidal to globose with muriform septa, 4.1–9.8 µm × 2.8–3.5 µm

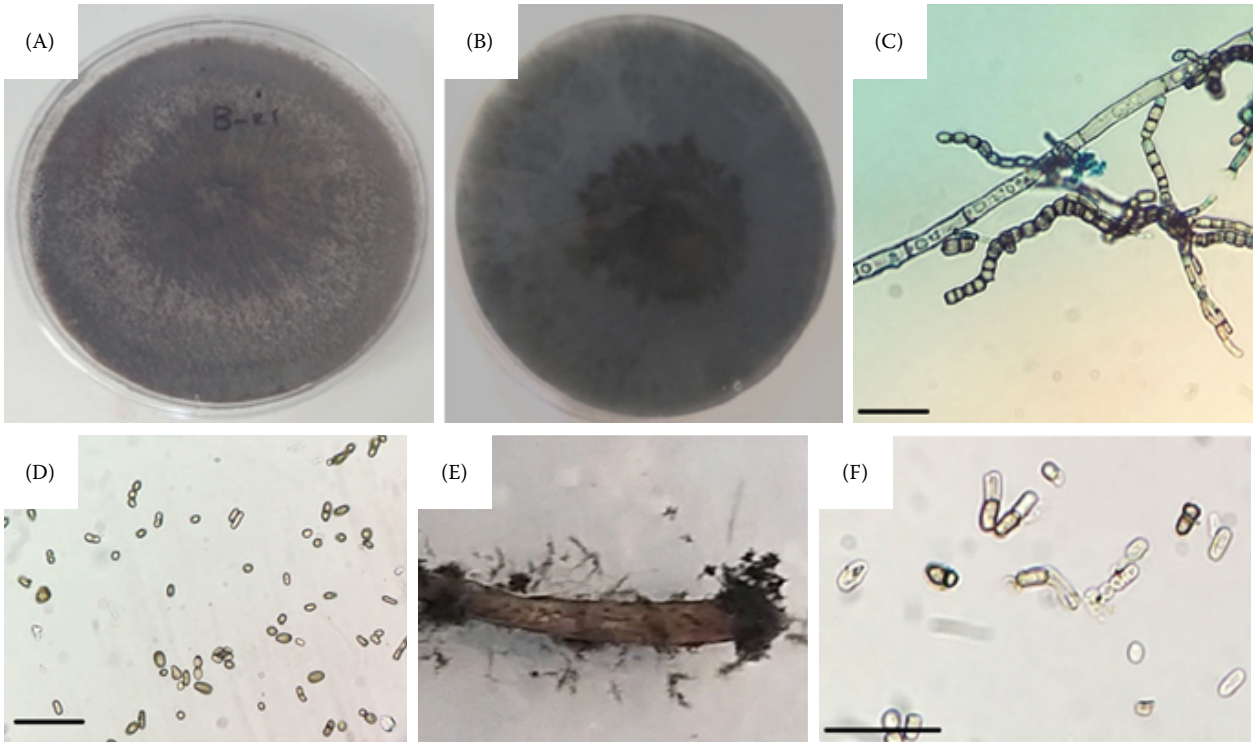

Figure 2. Morphology characters of *Neoscytalidium dimidiatum* isolate B-21 (Syn. *N. hyalinum*)
(A, B) Colony on potato dextrose agar; (C, D) mycelium and arthoconida; (E) conidiomata on pine needles on potato dextrose agar culture; (F) the conidia. Scale bar: C, D = 15 µm; F = 20 µm

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(Figure 3). According to morphological and cultural characteristics, the fungus was identified tentatively to the *N. dimidiatum* (Syn. *N. novaehollandiae* Pavlic, T.J. Burgess, & M.J. Wingf) (Philips et al. 2013). The subcultures were purified from single conidia of one isolate prepared on PDA. Representative culture of the *N. dimidiatum* was deposited in Mycology bank, Plant Protection Department, College of Agricultural Engineering Sciences, Salahaddin University-Erbil University, Kurdistan region, Iraq with isolates name B-20 and B-22.

Pathogenicity tests. The typical symptoms of stem canker developed from the site of inoculation, *Neoscytalidium* isolates could cause severe infection, resulting in necrosis streaks along barks and xylem of the inoculation points. This implies the black layer of fungal growth that cause progressive dieback and black discoloration to xylem tissues in eucalyptus and chinaberry seedlings (Figures 4 and 5). In addition, fallen leaves were noted along with dieback. No symptoms were noted on the uninoculated plants. However, after six months of inoculation, isolates of *N. dimidiatum* (B-21) develop wounds that are somewhat longer (28 mm and 55 mm in eucalyptus and chinaberry) than *N. dimidiatum* isoalte B-22 (25 mm and 40 mm respectively). Re-isolation of both *Neoscydalidium* isolates from the diseased tissues confirmed Koch's postulate.

Molecular characterization. ITS, LSU, and Bt2a regions were obtained for all studied isolates to confirm the identification based on morphology. Specific primers were designed by using the sequences of ITS, LSU, and Bt2a genes, which are synthesized by Microgene company (Korea). The primers could yield a band of the expected size for ITS gene 650 bp, LSU gene 1 200 bp and Bt2a gene 495 bp. The PCR product was electrophoresed and visualized by 1.5% agarose gel. The primers were found to produce expected band sizes.

Partial 28S rRNA and *Bt2a* sequenced gene. DNA, which was used only forward primers, was sequenced separately by ABI 3130X genetic analyzer (Applied Biosystems, USA). The PCR products were used for the fungus samples as a source of DNA template for sequence specific PCR amplification. All three sequences ITS, LSU and Bt2a were submitted to the NCBI under accession numbers as shown in Table 3.

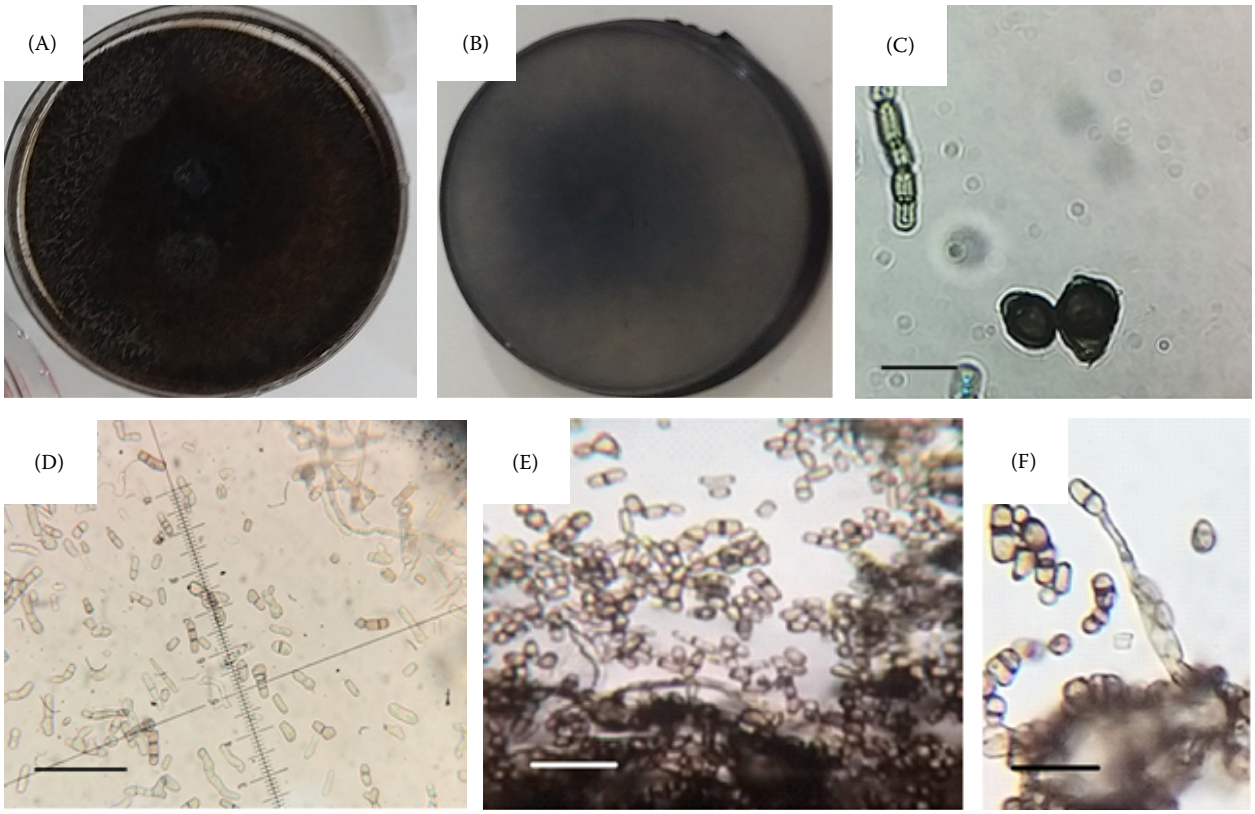

Figure 3. Morphology characters of *Neoscytalidium dimidiatum* isolate B-22 (Syn. *N. novaehollandiae*)
(A, B) Colony on potato dextrose agar; (C) muriform conidia; (D, E, F) arthrospores. Scale bar = 20 µm

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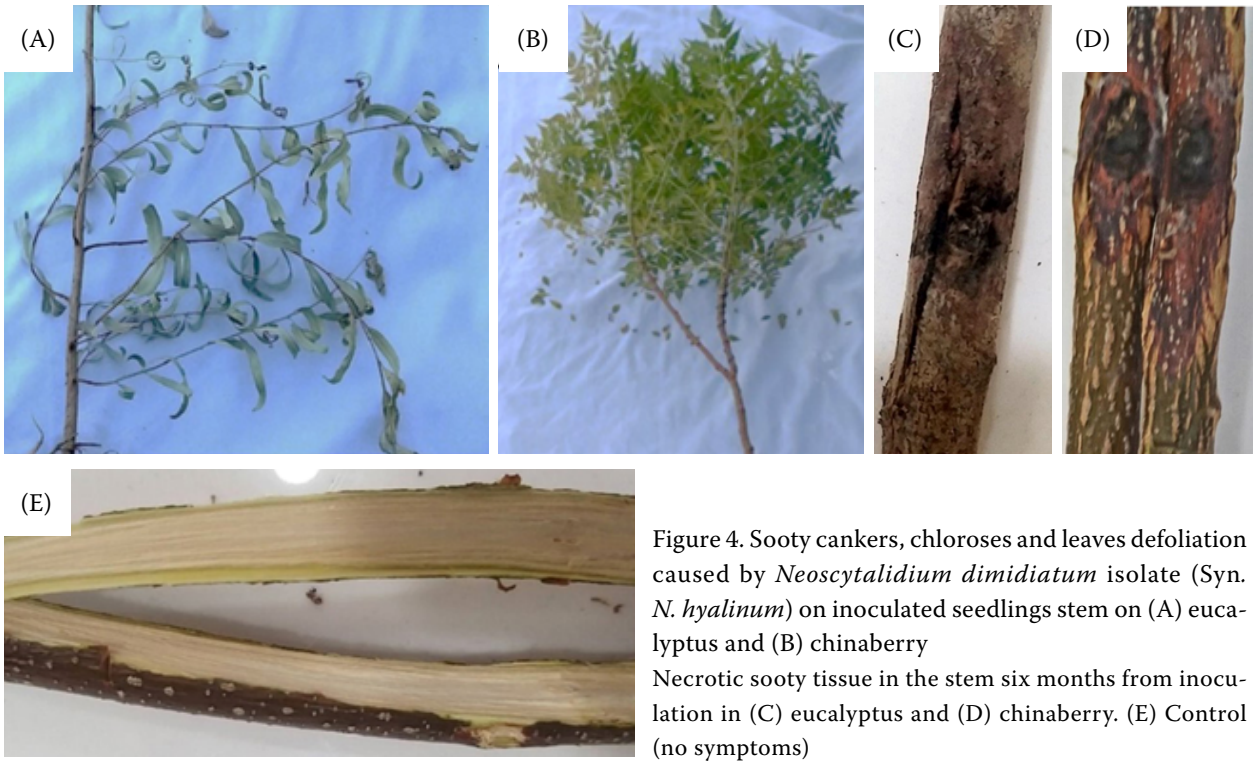

Figure 4. Sooty cankers, chloroses and leaves defoliation caused by *Neoscytalidium dimidiatum* isolate (Syn. *N. hyalinum*) on inoculated seedlings stem on (A) eucalyptus and (B) chinaberry
Necrotic sooty tissue in the stem six months from inoculation in (C) eucalyptus and (D) chinaberry. (E) Control (no symptoms)

Figure 5. Sooty cankers, chloroses and leaves defoliation caused by *Neoscytalidium dimidiatum* isolate (Syn. *N. novaehollandiae*) on inoculated seedlings stem of eucalyptus (A) and chinaberry (B)
Necrotic sooty tissue in the stem six months from inoculation in (C) eucalyptus and (D) chinaberry. (E) Control (no symptoms)

The combined sequences of the *N. dimidiatum* were formed well-supported clades according to the phylogenetic tree. Combined ITS, LSU and Bt2a datasets by Maximum Likelihood analysis were clade *N. hyalinum* (No. B21) and *N. novaehollandiae* (No. B22) together with *N. novaehol-*

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Table 3. GenBank accession No. of identified fungus in this study

Samples codes	Accession No.			Identified fungus *Neoscytalidium dimidiatum* isolates
	ITS	LSU	Bt2a	
B-18	MZ363622	–	–	*Neoscytalidium hyalinum*
B-20	OM278645	–	–	*Neoscytalidium novaehollandiae*
B-21	MZ363623	OM278779	OM320802	*Neoscytalidium hyalinum*
B-22	MZ363624	OM278780	OM320801	*Neoscytalidium novaehollandiae*

Bt2a – beta tubulin 2a; ITS – internal transcribed spacer; LSU – nuclear ribosomal large subunite

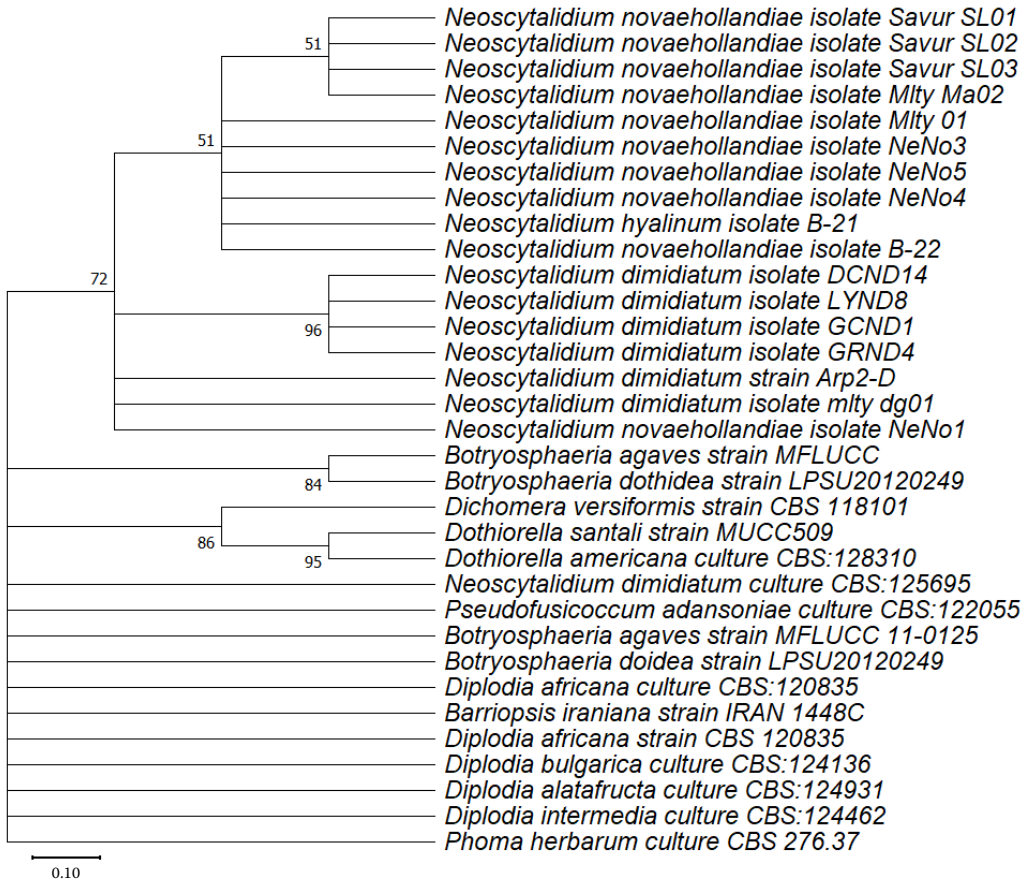

Figure 6. Phylogenetic maximum likelihood analysis of combined ITS, LSU and Bt2a generated in MegaX using Jukes-Cantor model and 1 000 replication bootstrapping for the new species *Neoscytalidium hyalinum* (No. B-21) and *N. novaehollandiae* (No. B-22) during this study

The tree was rooted to *Phoma herbarum*

landiae No. NeNo1, NeNo3, NeNo4, NeNo5, Milty ma 02, Savur SL01, Savur SLo2 and Savur SL03) (Figure 6). The Maximum parsimony analysis of three primers datasets together of both species was grouped together with *N. novaehollandiae* No. NeNo3, NeNo4, NeNo5, Milty ma 02, Milty 01, Savur SL01, Savur SLo2 and Savur SL03) (Figure 7).

DISCUSSION

Infected samples of eucalyptus and chinaberry trees possessing symptoms of stem cankers, shoot blight, and twig blight were collected from a different location in the Kurdistan region of Iraq. *Neoscytalidium* species were isolated from the infected plant samples. According to phylogenetic

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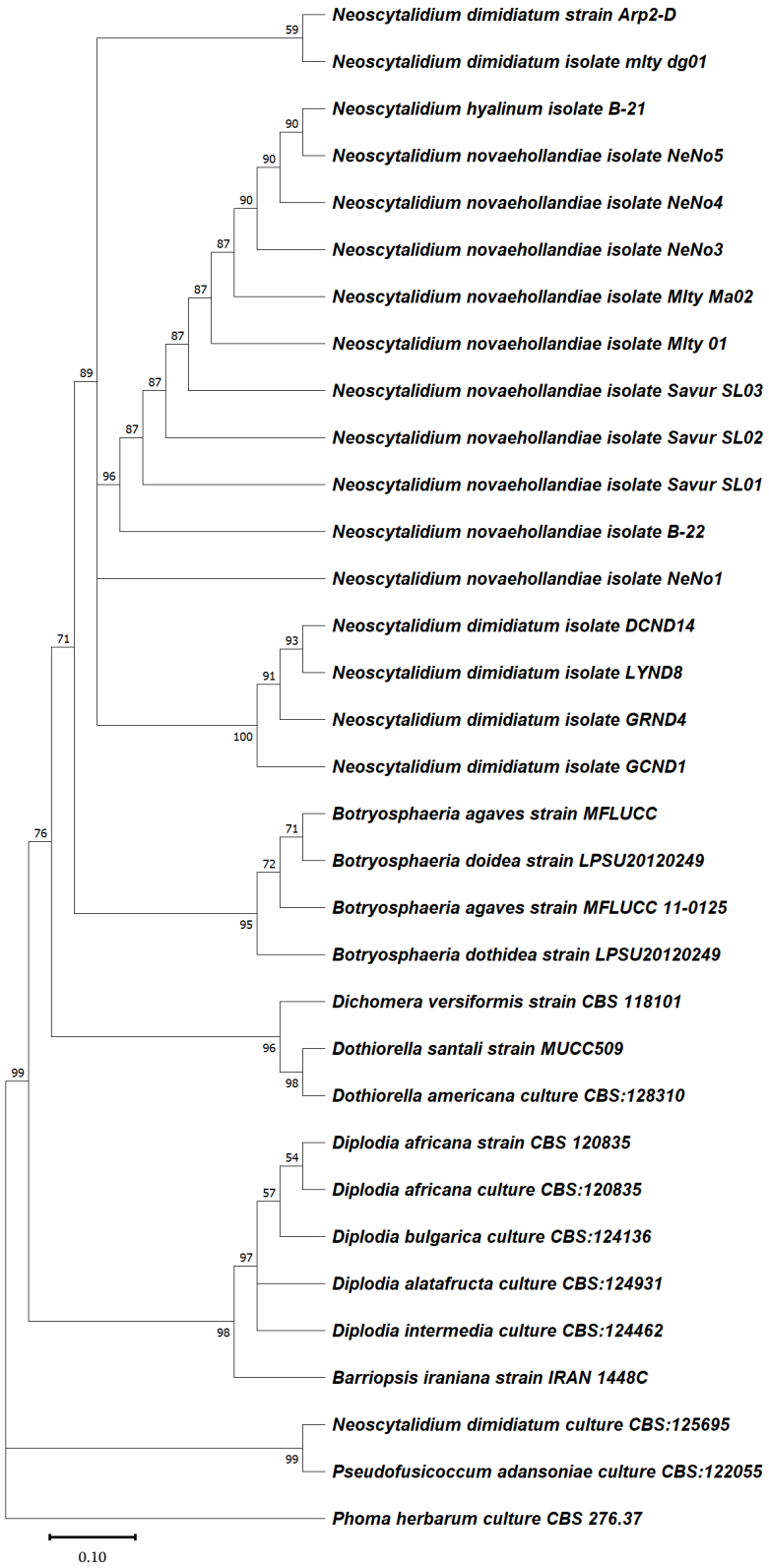

Figure 7. Phylogenetic maximum parsimony analysis of combined ITS, LSU and Bt2a generated in MegaX using Jukes-Cantor model and 1 000 replication bootstrapping for the new species *Neoscytalidium hyalinum* (No. B-21) and *N. novaehollandiae* (No. B-22) during this study
The tree was rooted to *Phoma herbarum*

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analyses and morphological characteristics, two *N. dimidiatum* isolates were identified as *N. hyalinum* and *N. novaehollandiae* among a large collection of isolates. Pavlic et al. (2008) illustrated that *N. novaehollandiae* produces muriform and *Dichomera* conidia, which separate it from other *Neoscytalidium* species.

In this study, ITS, LSU, and Bt2a sequences were used to identify and describe species of *Neoscytalidium*. However, polymorphic nucleotides occurred between some isolates in this work and other related species according to phylogenetic analyses. Phillips et al. (2013) and Chen et al. (2015) also used the ITS, LSU, and Bt2a sequences to classify and describe the Botryosphaeriaceae species. The ITS regions of the genomic ribosomal RNA gene were previously applied successfully for discriminating *Neoscytalidium* species (Ray et al. 2010b).

Furthermore, inoculation experiments indicated that the identified species of Botryosphaeriaceae in the study were pathogenic to eucalyptus and chinaberry trees. The outcomes are similar to previous studies confirming that Botryosphaeriaceae species are pathogens to *Eucalyptus* species (Pavlic et al. 2008; Chen et al. 2011). Mohali et al. (2009) showed that some Botryosphaeriaceae species are more aggressive than others on infection *E. urophylla* and *E. grandis*, indicating the resistance of different genotypes of *Eucalyptus* might be significantly different. Therefore, identifying resistant *Eucalyptus* genotypes to Botryosphaeriaceae will promote selecting resistant genotypes for planting.

The results of pathogenicity tests by Sabernasab et al. (2019) demonstrated that all 14 isolates of *N. novaehollandiae* are pathogenic to *Quercus brantii* transplants. Fifty-five days after inoculation, cankers were visible on the stems of all the inoculated seedlings and extended both upward and downward at the inoculation points. In addition, they showed yellowing, withering, and internal necrosis. Furthermore, Al-Bedak et al. (2018) carried out a pathogenicity test for *N. dimidiatum* based on artificial inoculations, which resulted in dieback symptoms in ficus plants. The signs were developed to 80% on *Ficus benjamina*, peeling the periderm of the inoculated plants showed the existence of a black layer of fungal growth. Alizadeh et al. (2022) stated that pathogenicity tests on chinaberry's detached branches and fruits showed brownish chlorotic lesions and chlorotic tissues around the inoculation site with black spores after forty days of inoculation. *N. dimidiatum* has already been found in a wide variety of woody plants all over the world (Punithalingam & Waterston 1970; Sutton & Dyko 1989; Crous et al. 2006). *N. dimidatum* causes diseases in several plant species, including apricot tree decline in Tunisia (Namsi et al. 2010), olive twigs and branch die back in California (Úrbez-Torres et al. 2013), ficus canker in Egypt (Al-Bedak et al. 2018), root rot of sweet potato in Brazil (Mello et al. 2019), pine shoot and needle blight in Turkey (Turkolmez et al. 2019), dragon canker in China (Xu et al. 2018), and citrus shoot blight in Jordan (Alananbeh et al. 2020). The *N. dimidiatum* infection process includes the formulation of an appressorium, direct cuticle penetration, and colonization of the underlying epidermal cell. A toxin playing a role in pathogenesis is consistent with the rapid clearing of cytoplasm, softening of cell walls, and collapse of tissue around and below the infection. Similar to this, the absence of hyphae and loss of cellular structure in the tissues of the yellow halos point to a diffusible toxin that was killing host tissue even before the pathogen invasion. Investigating protein variation during pathogen infection can be done using the powerful and effective method of quantitative proteomics (Li et al. 2018). Recently, a number of proteins were discovered to be involved in host infection, including pathogenicity-associated proteins and proteins involved in the breakdown of plant cell walls and redox regulation (Kim et al. 2017).

CONCLUSION

Neoscytalidium species are widely dispersed over the various geographical regions of Kurdistan – Iraq. ITS, LSU, and Bt2a are effective primers for the fast and reliable identification of *Neoscytalidium* species associated with cankers in chinaberry and eucalyptus trees. This is the first record of *N. dimidiatum* on chinaberry and eucalyptus trees in Iraq using three primers ITS, LSU and Bt2a. Extreme climate change in the Kurdistan region and stress factors may contribute to eucalyptus and chinaberry trees' susceptibility to dieback and stem canker diseases associated with *Neoscytalidium* species. Strong breeding programs

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that select chinaberry and *Eucalyptus* genotypes suitable to the climate, soil, and silvicultural techniques (spacing and thinning) should be carried out for disease management as part of an integrated management strategy. Further research is required to understand these species' biology and epidemiology and their populations' genetic diversity to manage the disease.

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Received: August 4, 2022
Accepted: December 28, 2022
Published online: January 31, 2023