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Full Length Research Paper

First report of grapevine dieback caused by *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum* in Basrah, Southern Iraq

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In Basrah, grapevines suffer from dieback. *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum* were isolated from diseased grapevines, *Vitis vinifera* L. and identified based on morphological characteristics and DNA sequence data of the rDNA internal transcribed spacer (ITS) region. The results of the pathogenicity test conducted under greenhouse conditions for *L. theobromae* and *N. dimidiatum* reveal that both species were the causal agents of grapevines diebacks in Basrah, Southern Iraq. A brief description is provided for the isolated species.

Key words: grapevine, dieback, *Lasiodiplodia theobromae, Neoscytalidium dimidiatum*, internal transcribed spacer (ITS), rDNA, Iraq.

INTRODUCTION

Grapevine, *Vitis vinifera* L. is the most widely planted fruit crop worldwide and is cultivated in all continents except Antarctica (Mullins et al., 1992). The area under plantation in iraq is 240,000 hectares, and it has an annual production of 350,000 tons grape (FAO, 1996). It is an economically important fruit crop globally. In Basrah Province, Southern Iraq, grapevines are mainly cultivated in the Abo-Alkasib area which is located near Shatt Al-Arab River and dominated by date palm plantations.

In a survey of grapevines grown in Basrah, several vineyards were found to suffer from dieback disease that killed the plants. The water salinity of the Shatt Al-Arab at Qarmatt Ali was found to be approximately 0.5 p.p.t but in recent years, it was noted to be more than 2 p.p.t. Several contributing factors to the increase in salinity are likely to have been the great reduction in levels of salts flushed out, continuous flushing of salts from irrigated land via drainage canals into the river and the linkage of the southern part of Haur Al-Hammar (at Qarmat Ali) to a new canal, the “Al-Basrah Canal”, which runs parallel to Shatt Al-Arab river into the Arabian Gulf (Bedair, 2006). Many environmental stress factors weaken plant hosts and make them more susceptible to infection by fungi. Out of these factors is the salinity which may contribute to reduction in plant growth through the effect on physiological functions such as photosynthesis, respiration and absorption (Bernstein and Hayward, 1958; Leonova and Shevyalova, 1970; Alsaidi, 2000), and increase the vulnerability to organism. Reckhaus (1987) considered that stress factors enhanced the severity of dieback disease caused by *Botryosphaeriaceae* species.

Species of the *Botryosphaeriaceae* are economically important opportunistic pathogens, as well as being saprophytes on decaying plant materials, whereas others are entirely endophytic (Barr, 1987; Smith et al., 1996; Denman et al., 2000). Many of these fungi cause canker and dieback diseases on numerous plant hosts throughout the world (von Arx, 1987; Burgess et al., 2006; Slippers and Wingfield, 2007). The taxonomy and identification of *Botryosphaeriaceae* is mostly based on anamorphic descriptions, because the teleomorphs are ob-

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observed rarely in nature and seldom produced in culture (Jacobs and Rehner, 1998; Denman et al., 2000; Zhou and Stanosz, 2001; Phillips, 2002; Crous et al., 2006; Schoch et al., 2006). In the recent study, on the basis of 28s rDNA sequence data and multi-gene approach, 16 genera were related to *Botryosphaeriaceae* (Crous et al., 2006; Damm et al., 2007; Phillips et al., 2008). The anamorphic states of this group, including *Diplodia, Lasiodiplodia, Neofusicoccum* and *Neoscytalidium* have been reported from infected grapevines as causal agents of cankers, dieback, bud mortality and bunch rot (Phillips, 2002; van Niekerk et al., 2004; Luque et al., 2005; Taylor et al., 2005; Úrbez-Torres et al., 2006a, 2008). Species of *Botryosphaeriaceae* have been reported to cause various dieback symptoms in grapevines. These symptoms include wood streaking, shoot dieback, bud necrosis, cane bleaching and graft failure (Lohoczky, 1974; Larignon et al., 2001; Phillips, 2002; Taylor et al., 2005; Úrbez-Torres et al., 2008a, b). The symptoms are characterized by slow development and their severity increased with age of the grapevines which are 8 or more years old or that are subjected to stress (Boyer, 1995; Larignon and Dubos, 2001). Some of the symptoms, especially brown wood streaking, resemble those found in trunks and arms affected by Petri disease and esca complex caused by *Phoeomoniella chlamydospora* (Mugnai et al., 1999; Phillips, 2002). Various species in this family can be isolated from grapevine and are only weakly pathogenic or entirely non-pathogenic (Phillips, 2002).

Lasiodiplodia theobromae (Pat.) Griffon and Maubl., is a common plant pathogen in the tropical and subtropical areas of the world. It is associated with various diseases including cankers, dieback and root rot on a wide range of hosts (Punithalingam, 1980). Vascular cankers and grapevine dieback caused by *L. theobromae* were first reported in Egypt in 1972 (El-Goorani and El-Meleigi, 1972). After 15 years, a field study carried out in California showed *L. theobromae* to be an important grapevine pathogen (Laevitt and Munnecke, 1987). Recent studies showed that the fungus has been a significant pathogen associated with decline of grapevine in South Africa and Australia (van Neikerk et al., 2004; Taylor et al., 2005; Wood and Wood, 2005). A study conducted in vineyards of Sonora and Baja, California revealed that perennial cankers and consequent grapevine dieback caused by *L. theobromae* and *Diplodia seriata* were a major problem in the most important grape-production areas of Mexico (Úrbez-Torres et al., 2008). Canker of grapevine caused by a *Botryosphaeriaceae* species was first reported in Sicily, and the first report on infections caused by *L. theobromae* on grapevine was in Italy (Burruano et al., 2008).

Neoscytalidium was introduced by Crous and Slippers as a new genus in the *Botryosphaeriaceae* and this type of species is designated as *Neoscytalidium dimidiatum* (Penz.) Crous and Slippers, comb. nov. (Crous et al., 2006). *N. dimidiatum* has been reported on diverse woody plants, frequently as *Hendersonula toruloidea* (Punithalingam and Waterston, 1970; Sutton and Dyko, 1989; Farr et al., 2004). Although, reported to be cosmopolitan, the disease caused by this fungus tend to occur in tropical countries as well as California (Farr et al., 2005). Symptoms include gummosis and dieback of stone fruit trees in Egypt (Nattrass, 1933), branch wilt, decline and death of citrus in Iran (Alizadeh et al., 2000), leaf spot diseases in India (Chandra, 1974) and a canker and a dieback of *Eucalyptus* in Arizona (Matheron and Sigler, 1993). So far, little attention has been given to mycotic grapevine diseases in Iraq. Earlier research by Natour and Ahmed (1969) showed that *Hendersonula toruloida* caused branch wilt of grapevines in the central region of Iraq. Recently, Saido (2007) isolated some fungi associated with grapevine decline in Duhok Province, Northern Iraq, including particularly *Botryosphaeria* spp., *Phaeoacermonium aleophilum* and *Cylindrocarpon destructans.* These represented new records for Iraq. Therefore, this is the first report on fungi grapevine diseases in Basrah region, Southern Iraq.

MATERIALS AND METHODS

Symptomatic grapevine parts (arms, canes and leaves) were collected from several vineyards in Abo-alkasib area, Basrah Province, Southern Iraq. Small pieces of infected tissue taken from the margin between necrotic and apparently healthy tissue, were surface sterilized with 5% sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. The surface- sterilized pieces were placed in Petri dishes lined with sterilized, moist filter paper and 10 pieces were placed in Petri plates containing potato dextrose agar (PDA) (Himedia Laboratories Pvt. Ltd., india) supplemented with chloramphenicol (50 µg/ml) to prevent bacterial growth. All plates were incubated at 25°C until fungal propagules were observed. Pure cultures of each isolate were obtained by excising a hyphal tip onto plates of potato dextrose agar. In order to enhance sporulation, cultures were placed on 2% water agar bearing pieces of autoclaved grapevine canes and inoculated at 25°C. Isolates were examined weekly for the formation of pycnidia and conidia. Isolated fungi were identified based on the morphological characters in culture and on natural substrates.

Molecular phylogenic characterization

DNA isolation

Total genomic DNA was isolated from fresh mycelium according to a miniprep protocol described by Cenis (1992). Pure cultures of each isolate were inoculated onto 500 µl of liquid potato dextrose medium (Difco Laboratories Ltd., Surrey, United Kingdom) and incubated at 25°C for 72 h. The mycelial mat was pelleted by centrifugation at 13,000 rpm for 5 min and was washed with 500 µl of Tris-EDTA, then homogenized by hand in 300 µl of extraction buffer [200 mM Tris-HCl (pH 8.5) 250 mM NaCl, 25 mM EDTA and 0.5% sodium dodecyl sulfate] for 5 min. 150 µl of 3 M sodium acetate (pH 5.2) was added, and the mixture was cooled to 20°C for 10 min. Fungal debris was pelleted by centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 13,000 rpm for 10 min and washed with 70% ethanol, then air dried and resuspended in 100 µl TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). 10 mg/ml of RNase was added at 37°C

for 45 min to digest the RNA. The DNA was quantified using Eppendorf® BioPhotometer and was stored at -20°C.

PCR amplification of ribosomal DNA regions

The universal primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS/5.8S coding rDNA gene regions as described by White et al. (1990). Each PCR reaction mixture contained 5 ng of genomic DNA, 1 µM of each primer, PCR reaction buffer [50 mM KCl, 50 mM Tris-HCl; (pH 8.3) 0.1 mg/ml bovine serum albumen], 3 mM $MgCl_2$, 200 µM of each dNTP, 2.5 U of Taq DNA polymerase (Promega) and autoclaved d_2H_2O to make-up the total volume of 50 µl. The PCR protocol consisted of an initial denaturation step at 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 54°C for 30 s and 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide and viewed with ultraviolet light. PCR amplified fragments were purified using QIAquick PCR (Qiagen, Amersham, Piscataway, NJ, USA) columns following the manufacturer's instructions and negative control (PCR reagent mixture) was included in the amplification.

DNA sequencing

PCR products were purified using the Promega gel and PCR clean-up system. The instructions of the QIAquick Gel extraction kit (QIAGEN) were followed and directly cycle sequenced in both directions using the BigDye Ready Reaction Kit (PE Applied Biosystems, Foster City, California) on an ABI Prism automated DNA sequencer (model 377, version 2.1.1; Applied Biosystems Warrington, United Kingdom) with the same primers.

Phylogenetic analysis

The sequence results from all samples when compared with DNA database demonstrated that they were derived from the fungal ITS regions. To identify the species, a sequence similarity search was performed using BLAST (Basic Local Alignment Search Tool) at NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The most similar 100 sequences were downloaded from NCBI and aligned using ClustalX2. The phylogenetic tree was viewed using MEGA 5.0.

Pathogenicity test

Pathogenicity tests were made on 2 years old grapevine plants of the Abbassi cultivar. In each experiment, apparently healthy looking plants were selected and specimens were taken from their arm, cane and leaves to confirm the absence of the test pathogens. Plants found infected with the test pathogen(s) were not used in the study. A cut in the arm, cane and leaf was made using a sterile sharp blade and inoculated by spraying either with 40 ml of conidial suspension (5 x 10^6 conidia ml^{-1}) (as above) or sterile water (controls) using an atomizer. The conidia was quantified microscopically using a Neuberger chamber and adjusted to 10^6 conidia/ml with d_2H_2O containing 0.02% Tween 20. Plants were monitored for one week to observe the development of disease symptoms. To satisfy Koch´s postulates, small pieces of necrotic tissue were cut from the edge of each lesion, surface-sterilized for 5 min with 5% sodium hypochlorite, washed three times with sterile distilled water, placed on PDA Petri plates, and then incubated at 25°C. After 4 days of inoculation, small pieces/tissues were examined for lesions/disease symptoms.

RESULTS AND DISCUSSION

Morphology

Two organisms were consistently isolated from plant parts showing dieback symptoms. Based on the morphological characters, isolates from the Basrah Region of Southern Iraq were identified as *L. theobromae* and *N. dimidiatum*. The identification of the isolates was performed using the relevant publications: Ellis (1971), Punithalingamm (1976, 1980), Sutton and Dyko (1989), Crous et al. (2006) and Alves et al. (2008). Colonies of *L. theobromae* which were white became dark olivaecious with dense aerial mycelium (Figure 1A). Pycnidia formed on grapevine canes after 20 days in culture were dark brown to black (Figure 1B). Conidiogenous cells hyaline had cylindrical hyaline and aseptate paraphyses (Figure 1D). Conidia were initially hyaline and became dark brown with one septa when mature with longitudinal striations of 17-23 x 10-12 µm (Figure 1C and D). This is the first record of this fungus as a pathogen of grapevines in Iraq. The climatic condition of Basrah city is mostly humid with high temperatures. These conditions might be favourable for infection by this pathogen. This result is in agreement with other studies (Punithalingam, 1980; Leavitt and Munnecke, 1987; Hewitt, 1988; Úrbez-Torres, 2006a, 2008; Burruano et al., 2008). This fungus has been isolated from grapevine showing decline and dieback symptoms in Egypt (El-Goorani and El-Meleigi, 1972), California (Leavitt and Munnecke, 1987), Arezona, Mexico (Leavitt, 1990), Portugal (Phillips, 1998, 2002), Australia (Castillo-Pando et al., 2001; Taylor et al., 2005), South Africa (van Niekerk et al., 2004), Spain (Úrbez-Torres, 2006b; Aroca et al., 2008; Martin et al., 2009), Argentina (Alves et al., 2008) and Bolivia (Kaiser et al., 2009). It was also reported on other hosts in adjacent countries, including mango in Oman and Pakistan (Adawi et al., 2003; Khanzada et al., 2004), coconut palms in India(Lakshman ana Jagadeesan, 2004), apricot and peaches in China (Li et al., 1995).

The hyphae of *N. dimidiatum* were mid to dark brown, pycnidia were dark brown to black and were formed on infected grapevine canes. Chains of arthroconidia were developed from unidentified, brown hyphae, conidia were cylinderical, brown, with 0 to 2 septate at maturity; 4-6 x 7-12 µm (Figure 2B). *N. dimidiatum* has been reported to cause branch wilt of grape in Baghdad, Central Iraq (Natour and Ahmed, 1969). This is the first time to record this fungus as pathogen of grapevine in Basrah province, Southern Iraq. *N. dimidiatum* has been reported from *Andansonia perrieri* in Australia (Sakalidis et al., 2011), citrus in Iran and Italy (Alizadeh et al., 2000; Polizzi et al., 2009), almond in California (Inderbitzin et al., 2010), *Prunus* spp. in Egypt (Farr et al., 2005) and mango in Niger (Pandey et al., 1981; Reckhaus and Adamous,

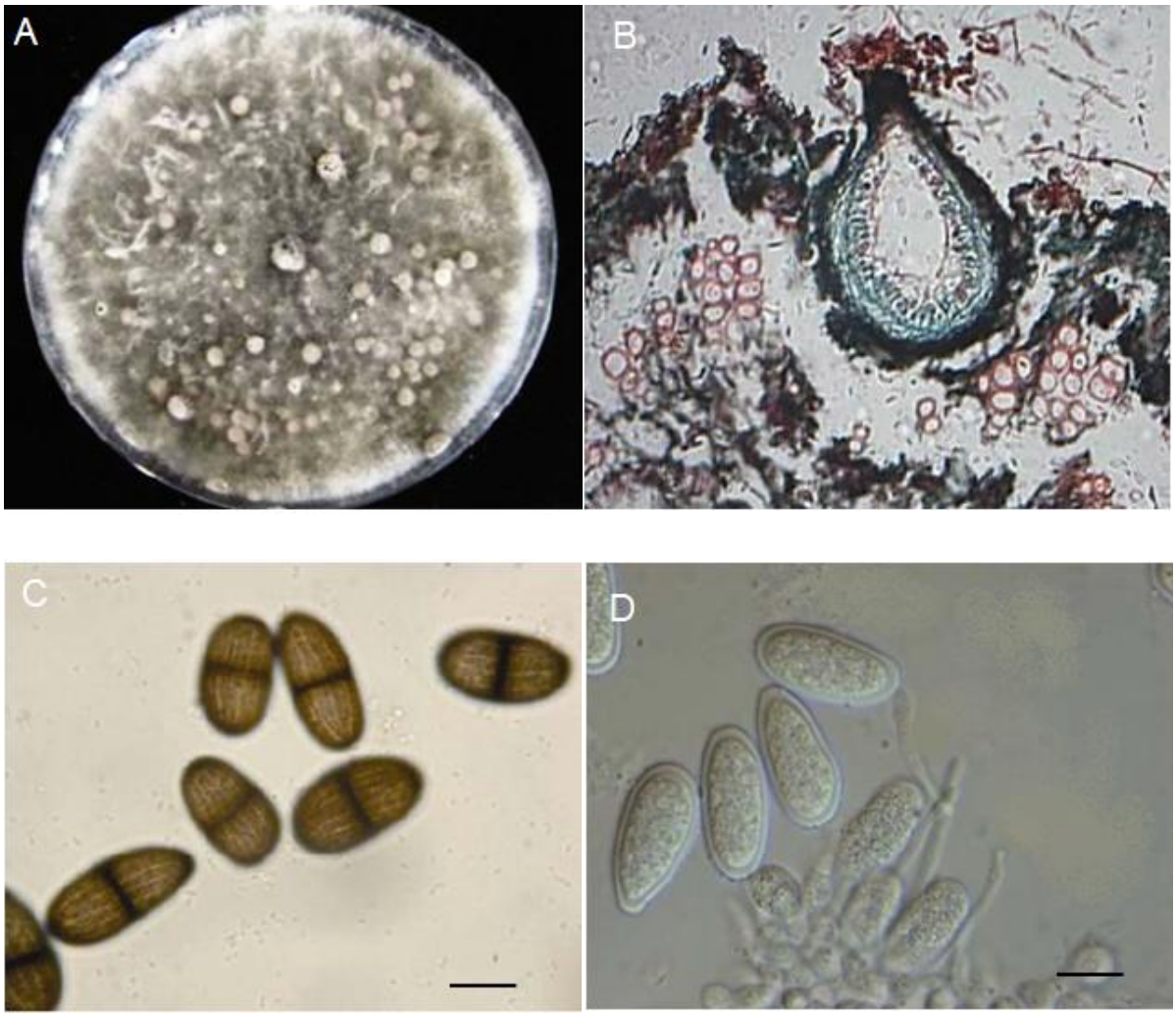

Figure 1. *L. theobromae*. A, Growth on potato dextrose agar (PDA) after 10 days; B, longitudinal section of infected shoot of grapevine showing pycnidium with 2-celled dark pycnidiospores; C, conidia mature dark brown with one septum; D, paraphyses cylindrical, aseptate, hyaline. Conidia initially produced hyaline and aseptate, subovoid to ellipsoid-ovoid, bar = 10 µm.

1987).

Phylogenetic analyses

The PCR-based method is considered as a quick and more sensitive technique as compared to morphological identification, with high discriminative ability between morphologically similar species.

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The ITS (the universal transcribed spacer) regions are typically variable and quite informative (Diaz and Fell, 2004). The universal fungal primers (ITS1/ITS4) successfully amplified the genomic DNA for all isolates that we tested. The sequence results demonstrated that they were derived from the fungal ITS regions when compared with the database of sequences on GenBank. BLAST results show that the most identical sequence was ITS/5.8S rDNA region of *L. theobromae* and *N. dimidiatum* showed 100% identity. The results of sequence similarity indicate that the isolated strains are *L. theobromae* and *N. dimidiatum*.

Pathogenicity tests

Grapevine plants that were inoculated with spore suspensions of *L. theobromae* and *N. dimidiatum* developed clear dieback symptoms after approximately 2 weeks (Figure 3A, C and D). No symptoms were observed on

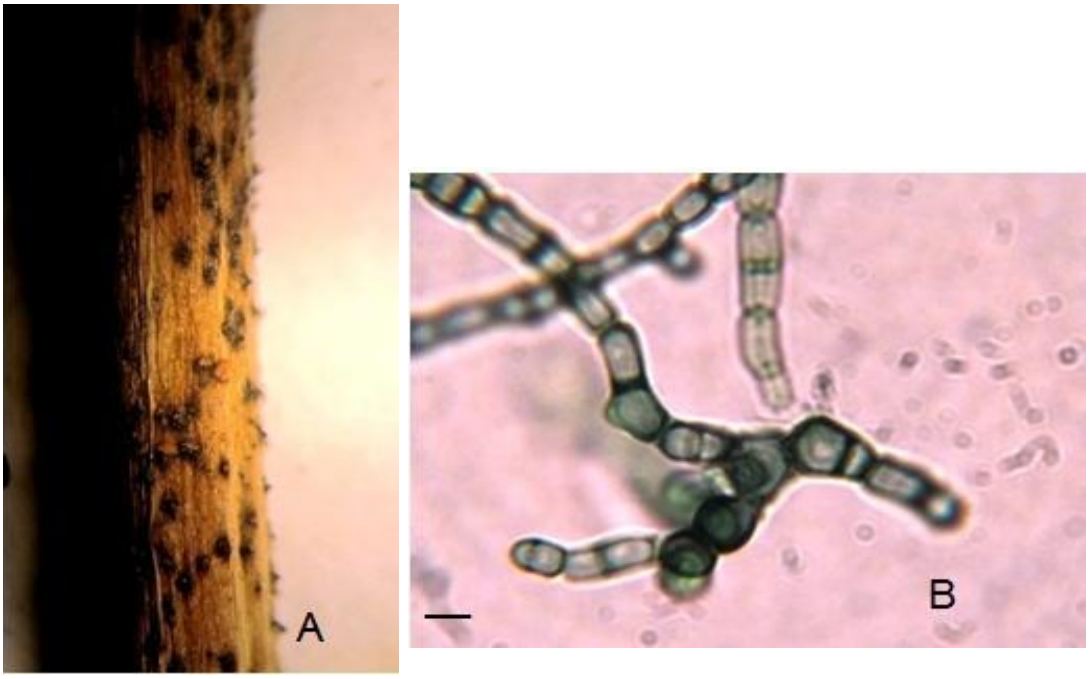

Figure 2. A, *L. theobromae*: Pycnidia dark brown to black formed on grapevine canes; B, *N. dimidiatum*: hyphae and chains of orthroconidia; bar = 4 µm.

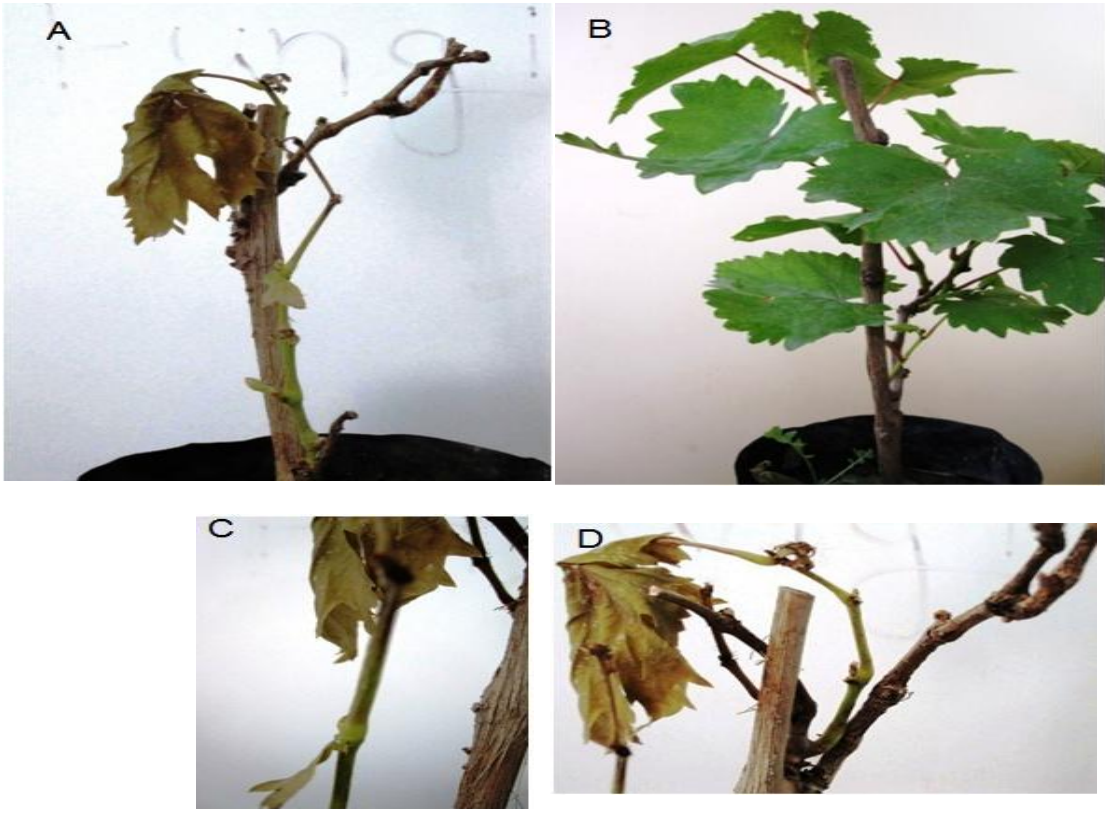

Figure 3. Grapevine plant inoculated with spore suspensions (A and D, *L. theobromae*; C, *N. dimidiatum*] developed clear dieback symptoms approximately 2 weeks after inoculation. Darkened wood or discoloration was observed in the green arms and canes; B, grapevine plant uninoculated with spore suspensions of *L. theobromae* and *N. dimidiatum.*

uninoculated plants (control) (Figure 3B).The most obvious symptoms on plants inoculated with *L. theobromae* and *N. dimidiatum* was the presence of dark wood or discoloration observed in the green arms and canes

During the trial period, there were no obvious symptoms of *L. theobromae* observed on leaves of inoculated plants. This agrees with previous studies conducted in California, Portugal and Mexico, which showed that there were no foliar symptoms associated with this pathogen (Phillips, 1998; Urbez-Torres et al., 2006, 2008). This study shows that *L. theobromae* was more virulent than *N. dimidiatum*. This result is in agreement with previous studies conducted in California, South Africa and Mexico in which *L. theobromae* was shown to be one of the most virulent species (Leavitt, 1990; van Niekerk et al., 2004; Úrbez-Torres, 2008). However, in Western Australia, *L. theobromae* isolates were avirulent or only weakly pathogenic in inoculated Red Globe mature canes (Taylor et al., 2005).

L. theobromae and *N. dimidiatum* may cause a serious dieback disease to vineyards in Iraq, particularly with the relation to the environmental conditions. Pycnidia of *L. theobromae* started developing on inoculated canes and trunk about two weeks after inoculation (Figure 3A, C and D). Plants inoculated with *N. dimidiatum* began to desiccate and lost their green color from the apex toward the base 7 days after inoculation. After four weeks, the entire seedling turned dark brown (Figure 3A, C and D). *L. theobromae* and *N. dimidiatum* were reisolated from the artificially inoculated plants, whereas no fungi were reisolated from the control treatments.

It is worth mentioning that accurate identification of *Botryosphaeriaceae* species is necessary to monitor global distribution of these pathogens, which contribute to appropriate application of quarantine decisions and development of an effective disease management strategies, because these species differ considerably in their interactions with different hosts and environmental conditions (Britton and Hendrix, 1986; Denman et al., 2003).

This work represents the first molecular detection and pathogenicity of *L. theobromae* and *N. dimidiatum* on grapevine in this part of the world and is still preliminary, and may be useful for further surveys to correlate *Botryosphaeriaceae* species with the different disease in vineyards of Iraq.

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