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

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ABSTRACT

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* revealed that both species were the causal agents of grapevines diebacks in Basrah, Southern Iraq.A brief description is provided for the isolated species .

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INTRODUCTION

Grapevine *Vitis vinifera* L. is the most widely planted fruit crop worldwide and is cultivated on all continents except Antarctica (Mullins *et al*.,1992).The area under plantation in iraq is 240,000 hectares,and generate an annual crop 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 as being more than 2 p.p.t.Several of the contributing factors for the increase in salinity are likely to have been the great reduction in levels, continuous flushing of salts from irrigated land via drainage canals into the river and the linkage of the of the southern part of Haur Al-Hammar(at Qarmat Ali) to a new canal, the "Al-Basrah Canal",which run 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 reduce 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 seve-

rity of dieback disease caused by Botryosphaeriaceae species.

Species of the Bortyosphaeriaceae 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 descripttions , because the teleomorphs are 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 studies on the basis of 18s rDNA sequence data and multi-gene approach,16 genera were releated 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 a 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 in a 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.,*2008b, 2008).The symptoms are characterized by the slow development and their severity increased with age of the grapevines that are 8 or more year old or that are subjected to stress(Boyer,1995; Larignon and

Dubos,2001).Some of the symptoms, especialy brown wood streaking, are 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 are only weakly pathogenic or entirely non-pathogenic (Phillips,2002).

Lasiodiplodia theobromae (Pat.) Griffon and Maubl.,is a common plant pathogen in the trpical 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 *Lasiodiplodia theobromae* first were reported in Egypt in 1972(El-Goorani and El-Meleigi, 1972) .After fifteen years, a field study carryed 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 declining 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 ,2008).Canker of grapevine caused by a Botryosphaeriaceae species first was reported in Sicily ,and the first reported of infections caused by *L.theobromae* on grapevine in Italy (Burruano *et al.,*2008).

Neoscytalidium was introduced by Crous and Slippers as a new genus in the Botryosphaeriaceae and the type species designated is *Neoscytalidium dimidiatum* (Penz.) Crous and Slippers,comb.nov.

(Crous *et al.*,2006). *Neoscytalidium 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 on 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. Early research by Natour and Ahmed (1969) reported 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, includes 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.

2.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 ^{0}C until fungal propagules were observed. Pure cultures of each isolate were obtained by excising a hyphal tip on to 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 ^{0}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.

2.1 Molecular phylogenic characterization

2.1.1 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 25ºC for 72h.The mycelial mat was pelleted by centrifugation at 13,000rpm for 5min and was washed with 500µl of Tris-EDTA,then homogenized by hand in 300 µl of extraction buffer (200 mM Tris-HCl [pH 8.5],250mM NaCl,25mM 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 13,000rpm for 5min, the supernatant transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 13,000rpm for 10min and washed

with 70% ethanol, then air dried and resuspended in 100 µl TE (10 mM Tris-HCl [pH 8.0], 1mM EDTA).10mg/ml of RNase was added at 37ºC for 45min.to digest the RNA. The DNA quantified using Eppendorf® BioPhotom-eter and was stored at -20ºC.

2.1.2 PCR amplification of ribosomal DNA regions

The universal primers ITS1-F (5'CTTGGTCATTTAGAGGAA GTAA-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 5ng of geno-mic DNA, 1µM of each primer, PCR reaction buffer (50mM KCl,50mM Tris-HCl;[pH 8.3] 0.1mg/ml bovine serum albu-men), 3 mM $MgCl_2$, 200µM of each dNTP, 2.5 U of Taq DNA polymerase (Promega) and auto-claved 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 separa-ted 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.

2.1.3 DNA sequencing

PCR products were purified using the Promega gel and PCR clean-up system . The instructio-ns of the QIAquick Gel extrac-tion 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.

2.1.4 Phylogenetic analysis

The sequence results from all samples when compared to 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 phylogenyetic tree was viewed using MEGA 5.0.

2.2 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 40 ml of conidial suspension (5 x 10^6 conidia ml^{-1})(as above) or sterile water (controls) using an atomizer .The conidia quantified microscopiclly 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 developpment of disease symptoms. To satisfy Koch´s postulates, small pieces of necrotic tissue were cut from the edge of each lesion,surface sterilized for 5min. with 5% sodium hypochlorite, washed three times with sterile distilled water and placed on

PDA Petri plates, then incubated at 25 °C. After 4 days of inoculation, small pieces/tissues were examined for lesions/ disease symptoms.

3.RESULTS AND DISCUSSION

3.1 Morphology

Two organism were consistently isolated from plant parts showing dieback symptoms. Based on the morphological characters, isolates from the Basrah Region of Southern Iraq were identifyed 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 *Lasiodiplodia theobromae* white ,becomign dark olivaecious with dense aerial mycelium (Fig.1,A). Pycnidia were dark brown to black formed on grapevine canes after 20 days in culture (Fig.1,B). Conidiogenous cells hyaline with cyliderical hyaline and aseptate paraphyses(Fig.1,D).Conidia were initially hyaline becoming dark brown with one septa when mature and longitudinal striations ,17-23x10-12µm(Figs.1,C.D) .This is first record of this fungus as a pathogen of grapevines in Iraq. The climatic conditions of Basrah city is mostly humid with high temperatures. These conditions might be favorable for infection by this pathogen. This result is agreed with other studies (Punithalingam,1980;Leavitt,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,1992), Califonai (Leavitt ,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 adjcent countries ,including mango in Oman and Pakistan (Adawi *et al.,*2003 ;Khanzada *et al.,*2004), apricot and peaches in China(Li *et al.,* 1995).

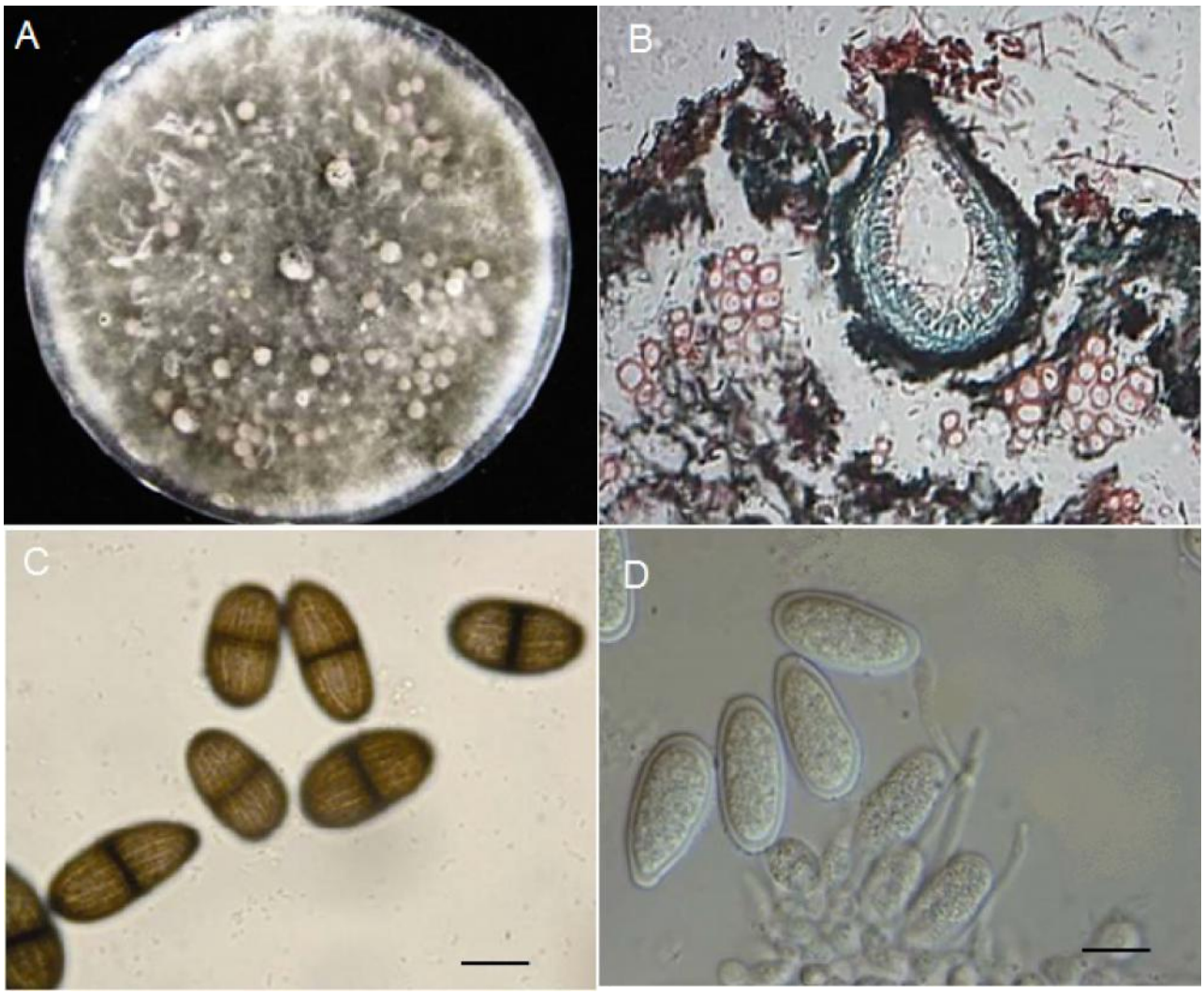

Fig.1.*Lasiodiplodia theobromae*.A.Growth on potato dextrose agar(PDA)after 10 days. B.Longitudinal section of infected shoot of grapevine showing pycnidium with 2-celled dark pycnidiospores C. Conidia mature dark brown with one septum D . Paraphyses cylindrical, aseptate, hyaline. Conidia produced initially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

The hyphae of *Neoscytaliduim dimidiatum* mid to dark brown, pycnidia were dark brown to black formed on infected grapevine canes. Chains of arthroconidia were developed from unidentified ,brown hyphae,conidia were cylinderical,brown,0-2 septate at maturity,4-6x7-12µm(Fig.2,B).

N. dimidiatum has been reported to cause branch wilt of grape in Baghdad, central Iraq (Natour & Ahmed, 1969).This is the first time to record of 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) and *Prunus* spp.in Egypt (Farr *et al.,*2005),mango in Niger (Pandey *et al.,*1981;Reckhaus and Adamous,1987).

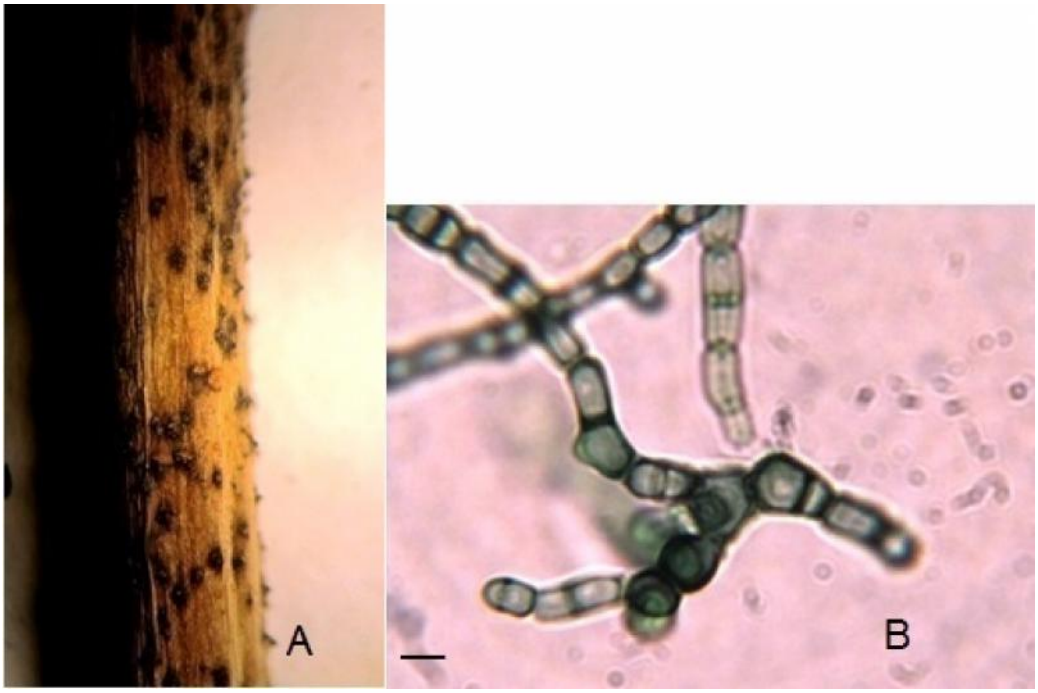

Fig.2. A. *Lasiodiplodia theobromae*.Pycnidia dark brown to black formed on grapevine canes.B. *Neoscytalidium dimidiatum*.Hyphae and chains of orthroconidia.Bar= 4µm

3.2 Phylogenetic analyses

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

The ITS (the universal transcribed spacer) regions are typically variable and quite informative (Diaz and Fell, 2004). The universal fungal primers (ITS1/ITS4) successfully amplifyed the genomic DNA for all isolates that we tested.

The sequence results demonstrated that they were derived from the fungal ITS regions when compared to the database of sequences on GenBank. BLAST results showed the most identical sequence was ITS/5.8S rDNA region of *L. theobromae* and *N. dimidiatum* showing 100% identity. The results of sequence similarity indicated that the isolated strains are *L. theobromae* and *N. dimidiatum*.

3.3 Pathogenicity tests

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

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, that found that there were no foliar symptoms associated with this pathogen (Phillips ,1998; Urbez-Torres *et al*. 2006, 2008). The present study showed that *L.*

theobromae was more virulent than *N. dimidiatum*. This result is in agreement with a 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 Autralia *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 create a serious dieback disease to vineyards in Iraq, particulary with the relationship to the environmental conditions. Pycnidia of *L. theobromae* started developing on inoculated canes and trunk about 2 weeks after inoculation (fig.3 A.C.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 4 weeks the entire seedling turned dark brown (fig.3, A, C, D). *Lasiodiplodia theobromae* and *N. dimidiatum* were reisolated from the artificially inoculated plants. Whereas no fungi were reisolated from the control treatments.

It was worth mentioning that accurate identification of botryosephaeriaceae species is necessary to monitor global distribution of these pathogens, contribute to appropriate application of quarantine decisions and develop 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 represent the first molecular detection and pathogenicity of *L.theobromae* and *N.dimidiatum* on grapevine in this part of the world and is still preliminary which may be useful for a further surveys to correlate

botryosphariaceae species with the different disease in vineyards of Iraq.

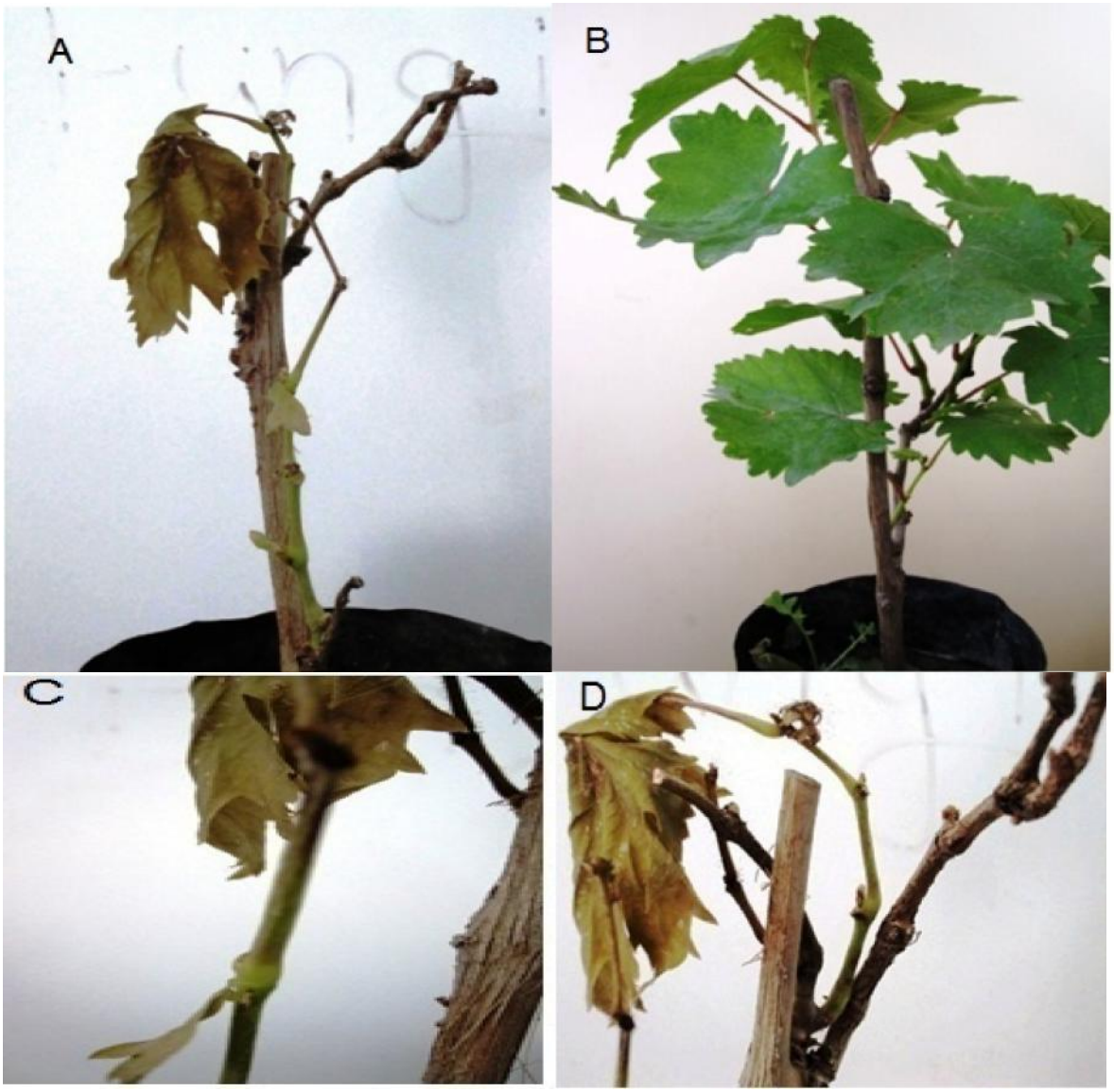

Fig. 3 .Grapevine plant inoculated with spore suspensions of [A.D. *Lasiodiplodia theobromae* C. *Neoscytalidium dimidiatum*] developed clear dieback symptoms approximately 2 weeks after inoculation. Darkened wood or discoloration was observed in the green arms and canes B. Grapevine plant uninoculated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

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اول تسجيل للفطرين *Lasiodiplodia theobromae* و *Neoscytalidium dimidiatum* كمسببين لمرض الموت الرجعي على العنب في محافظة البصرة جنوب العراق

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الخلاصة

تعاني بعض نباتات العنب .*Vitis vinifira* L في محافظة البصرة بجنوب العراق من مرض الموت الرجعي ،في هذه الدراسة تم عزل الفطرين *Lasiodiplodia theobromae* و *Neoscytalidium dimidiatum* من نباتات عنب ظهرت عليها اعراض مرض الموت الرجعي ،صنف الفطرين مظهريا و جينيا وبينت نتائج اختبار الامراضية ان الفطران سببا الموت الرجعي لنفس النبات عند التلقيح بهما، يعد تسجيل الفطر *L.theobromae* اول تسجيل له في العراق كممرض لنبات العنب بينما يعد تسجيل الفطر الثاني *N.dimidiatum* التسجيل الاول في البصرة كممرض لنبات العنب. .